Study of *Acinetobacter* species with special reference to drug resistance

Dissertation submitted to

The Tamil Nadu Dr. M.G.R. Medical University

In partial fulfillment of the regulations

For the award of the degree of

M.D. MICROBIOLOGY

DEPARTMENT OF MICROBIOLOGY

PSG INSTITUTE OF MEDICAL SCIENCES AND RESEARCH,

PEELAMEDU, COIMBATORE, TAMILNADU, INDIA

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COIMBATORE

CERTIFICATE

This is to certify that the dissertation work entitled **"Study of *Acinetobacter* species with special reference to drug resistance"** submitted by Dr. Santhosh Gandhi.S, is work done by him during the period of study in this department from September 2012 to January 2015. This work was done under direct guidance of Dr. B. Appalaraju, Professor and Head, Department of Microbiology, PSGIMS& R.

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CONTENTS

Introduction:

*Acinetobacer*acter species mainly *Acinetobacter baumannii* has emerged as an important and problematic human pathogen as it is the causative agent of several types of infections including pneumonia, meningitis, septicemia, and urinary tract infections. Acinetobacter species isolates are frequently resistant to multiple antibiotic classes through an array of resistant mechanism leading to multi and pan drug resistance. Characterization of antibiotic resistance genes in *A. baumannii,* is necessary to control further dissemination of these antibiotic resistant genes. Treatment of multidrug-resistant bacterial infections poses a therapeutic challenges, combination therapy is often the only viable option for multidrug-resistant and pandrug-resistant infections.

Aim:

Detection of genotypes of Carbapenamase present in multi and pan drug resistant *Acinetobacter baumanii* isolated from the clinical samples, and identify the best combination of drugs for effective and management of multidrug-resistant isolates.

1. **Material and Methods:**

Acinetobacter species were characterized by conventional phenotypic methods and vitek automated microbial identification system. Phenotypic and

genotypic methods were used to characterize the mechanisms of resistance in *Acinetobacter baumannii.i C*hecker board method was used to perform drug synergy test using Rifampin Amikacin Sulbactam Ciprofloxacin and Imipenem in combination with Colistin.

Result:

Acinetobacter baumannii was the most common species isolated in the hospital of which 73% were multidrug resistant and 50.02% were carbapenem resistant, MBL () AmpC() The molecular study showed OXA 51 was seen 100% and OXA 23 was in 93.05% . Better synergy was observed with rifampin (80%) and imipenem (77.77%) with colistin and poor synergy was seen with ciprofloxacin and colistin combination (10%).

Conclusion:

Acinetobacter baumannii is one of the important nococomial pathogen which showed high incidence of carbapenem resistance (58.03%) best synergy was observed with colistin and rifampin (80%) followed by imipenem (77.77%) most common OXA gene found in our study were OXA 51 and OXA 23.

INTRODUCTION

Acinetobacter species are non motile, oxidase negative coccobacilli, which is currently classified to *Moraxellaceae*[1]. It consists of at least 12 DNA hybridization groups referred to as genospecies. Genospecies 1 is the type species *A.calcoacticus* and was isolated from the soil. Genospecies 2 is *Acinetobacter baumannii* and is the most prevalent species in human clinical isolates[2-3].

Acinetobacter is a gram negative cocco-bacillary cell which often appears as diplcocci. After 24 hours of growth on blood agar, the colonies are between 0.5 and 2 mm in diameter translucent and opaque convex and entire. Most strains grow well in Mac-conkey agar which are pale lactose fermenting colonies. Presumptive identification of *Acinetobacter* species is made on the basis of the lack of cytochrome oxidase activity, lack of motility and resistance to penicillin. *A baumannii* is a saccharolytic and acidifies most *OF* carbohydrates; identification is made by demonstration of the rapid acid production of acid from lactose 1 & 10 % concentrations.

There has been extensive research done on the virulence factor of *Acinetobacter* but only little is known about the true pathogenic potential. The factors which contribute to its virulence include outer membrane proteins (OMPs)[11], biofilm formation that allows the bacteria to grow in unfavorable conditions with the help of the protein called BAP(biofilm-associated protein).[12] The other virulence factor include phospholipase D and C, that causes resistance in human serum and invasion of epithelial cell, while phospholipase C causes toxicity in epithelial cells[13]

A.baumannii is the species most prevalent in clinical specimens and most often responsible for hospital acquired infections. Other species such as *A.johnsonnii, A lwoffii* seem to be natural inhabitance of the human skin and may also be commensals in the oropharynx and vagina[3].A variety of human infections have been viewed by Lyons et al, including pneumonia, endocarditis, meningitis, skin and wound infections, peritonitis and urinary tract infection sporadic cases of conjunctivitis, osteomyelitis, and synovitis have also been reported[5]. They have been implicated in a various nosocomial infections, including bacteremia, urinary tract infection, secondary meningitis but their predominant role in ventilator-associated pneumonia confined to hospital intensive-care units.[6]

Acinetobacter species tends to be resistant to a variety of antibiotics, although one species, *A lwoffii* tends to be more sensitive than the others. There is almost universal resistance to penicillin, ampicillin and cephlothin and most strains are resistant to chloramphenicol[7]. It has been noticed that there is an increased trend for aminoglycosides resistance among the *Acinetobacter* species. In recent years multiple drug resistant strains, including carbapenems-resistant *Acinetobacter* species have been reported in nosocomial outbreaks.[8]

The resistance mechanisms for the various drug resistances in *Acinetobacter* are not studied extensively but found be similar to *Pseudomonas* spp[18]. The drug resistance mechanisms are based on the principle of

- Antimicrobial-inactivating enzymes, wide range of beta-lactamases that hydrolyze penicillins, cephalosporins and carbapenems[19].
- reduced access to bacterial targets,
- mutations that change targets or cellular functions[20]

Combination treatment with an aminoglycoside and ticarcillin or piperacillin is synergistic and may be effective in serious infections. For multiply resistant *Acinetobacter* infection several studies have demonstrated clinical efficiency of sulbactam in combination with ampicillin or cefoperazone. The other antimicrobial agent that has shown to be active against multiply resistant *Acinetobacter* is colistin[9-10].

Various studies have shown an in vitro evidence of heteroresistance among the colistin susceptible *Acinetobacter* isolates. The proportion of colistin resistant subpopulation is significantly increased by serial passages of the isolates in the presence of colistin. Combination therapy is advisable over monotherapy with colistin in order to prevent the emergence of heteroresistance[21].

Ventilator associated pneumonia is often associated with *Acinetobacter* due to the factors such as long period of hospitalization longer period on ventilators and use of broad spectrum of antibiotics which are considered to be some of the risk factors. In Intensive care unit the mortality rate was higher ranging between 26%-68% [14] and further increase in mortality rate in colonization[15]. In blood stream infections the mortality

was between 34%-43.4% in the intensive care unit and 16.3% outside the intensive care unit, *Acinetobacter* blood stream infections is the third highest cause of mortality followed only by *P.aeruginosa* and *candida* spp infections[16], but the alternate reason for the marked increase in mortality could be the patients' severe underlying illness and not an independent risk factor for mortality.[17]

In this study, we would like to speciate *Acinetobacter* isolates and study the effect of combination of drugs on multidrug resistant and pan drug resistant *Acinetobacter* which will help in the management of the patient with these infections.

We would also like to characterize the mechanism of resistance by studying OXA sub types.

2. AIM AND OBJECTIVES

AIM OF THE STUDY

Primary Objectives

Detection of genotypes of Carbapenamase present in multi and pan drug resistant *Acinetobacter baumanii* isolated from the clinical samples.

Secondary Objectives:

1. Isolation and characterization of *Acinetobacter* by conventional phenotypic methods and vitek automated microbial identification system.

2. Phenotypic detection of drug resistance mechanism by Modified Hodge Test and Combined EDTA disk test.

3. Drug synergy testing by checker board method using Rifampin Amikacin Sulbactam Ciprofloxacin and Imipenem in combination with Colistin.

4. REVIEW OF LITERATURE

REVIEW OF LITERATURE

HISTORICAL PERSPECTIVE *OFACINETOBACTER:*

Acinetobacter was first isolated by a Dutch microbiologist, Beijerinck from the soil using minimal media enriched with calcium acetate in the year 1911.[22] The name *Acinetobacter* was proposed later by Brisou and Prevot which was originally described as *Micrococcus calco-aceticus,* over 40 years ago when it was differentiated from the motile genus *Acromobacter.* It was accepted as a genus which could not be differentiated in to further species based on the phenotypical characters when an extensive study on the organism was done. In the committee on the Taxonomy of Morraxella and Allied Bacteria sub-committee officially accepted the genus *Acinetobacter* after the review based on the publication based on Baumann's 1968.

The current definition of *Acinetobacter* comprises gram negative, non fermenting strictly aerobic, non-fastidious, non motile, oxidase negative, catalse positive bacteria that has a DNA G+C content of 39% to 47% [23].Now there are 26 named species and nine genomic species which have phenotypic similarities which are difficult to

differentitate[24].*Acinetobacter* cannot be distinguished from other gram negative non fermenting bacteria by a definitive metabolic test while the species level identification for the 28 available phenotypes was proved to be 95.6%,the recently identified genomic strains have proven to be ineffective[25].It is now classified as the family *Moraxellaceae* order *gammaproteobecteria,* which include the genus *Acinetobecter*[26].

The isolates suspected to be *Acinetobacter* undergo routine phenotypetic test which is described by Gerner-Smid *et al* they are grown in 5% sheep blood agar at 37° and 44° C, using Hugh and leifson's medium which contain 1% glucose along with gelatin stab method to test for liquefaction. Then liquid media for assimilation test using carbon source with 0.1%concentration of malonate L-histidine t –Aconitate histamine and citrate are used for assimilation test Additional assimilation test such as DL - 4 aminobutyrate and l- phenylalanine the assimilation test are read after 1, 2 and 6 days the growth on the agar at 37° and 44° c on the second day except for gelatin liquification w hic is read at a temperature of 22°C

More molecular methods have been used to identify the species level of *Acinetobacter*[23].

- Amplified 16S rRNA gene restriction analysis (ARDRA).
- High-resolution fingerprint analysis by amplified fragment length polymorphism (AFLP).
- Ribotyping.
- tRNA spacer fingerprinting.
- Restriction analysis of the 16S–23S rRNA intergenic spacer Sequences.
- Sequence analysis of the 16S–23S rRNA gene spacer region.
- Sequencing of the rpoB (RNA polymerase β-subunit) gene and its flanking spacers.

Natural Habitats:

Acinetobacter can be isolated for all samples of soil and water hence called as ubiquitous organism. In a survey to study human skin flora of skin and mucous membrane it was found that up to 43% of the community had members of this organism. The most commonly found was *A.loffii* (58%), followed by *A.johnsonii*. The fecal carriage rate was found to 25%, the carriage rate in the wards of the hospital was found to be much

higher as 75%.[27] Based on the data it could be said that *A.johnsonii,* is found in soil water and on human skin.

A.lwoffii & A.radioresistense are found on human skin. The rate of carriage in Europe for *Acinetobacter baumannii* was found to be low, and in tropical countries, but found to be higher in countries like HongKong[28] Hence the habitat of *A.baumannii* is yet to be clearly understood.

CLINICAL MANIFESTATIONS OF *ACINETOBACTER baumannii* INFECTIONS:

Acinetobacter baumannii has been associated with broad spectrum of diseases in varying patient outcome and severity. There are quite a few studies that have concluded that infection with *A.baumannii* has a major effect on the patient outcome[29,30] while the other have implied very little or no impact on the patients outcome following the infection.[31,32]

COMMUNITY-ACQUIRED PNEUMONIA:

Community acquired pneumonia caused by *A.baumannii* has been found in tropical regions of Asia and Australia it has been typically described during the rainy season and history of alcohol abuse. The infection may be due to the reason that more than 10% of the population with the history of excess of alcohol drinking, a sever clinical course, secondary blood infection and mortality rate of 40 to 60%[33].

HOSPITAL-ACQUIRED PNEUMONIA:

Ventilator associated pneumonia is the commonly linked to the infection caused by *A.baumannii*. Ventilator-associated pneumonia occurs after the patient has been incubated and on mechanical support for more than 48 hours. It is very difficult to differentiate between airway colonization and pneumonia. Longer period of stay in the hospital, long periods on mechanical ventilation and prolonged use of broad spectrum antibiotics are found to be the risk factors that has been implicated in ventilator associated pneumonia caused by *A.baumannii*[34].

BLOODSTREAM INFECTION:

Acinetobacter was implicated as the cause of bloodstream infections in more than 1.3% of the cases in a seven year review done in United States[16]. When compared between the ICU and non ICU patients the infected with *Acinetobacter baumannii* the number of infection in ICU was significantly higher which was 43% and 16.5% respectively. The mortality rate was found to be third higher after *P. aeruginosa and candida* spp. In the review mentioned above the source of infection was not explained but could be attributed to underling urinary tract infection pneumonia and wound infection. In the same review of the 102 patient who were treated for the infection belonged to military people who were injured in Iraq of Afghanistan.

SKIN AND SOFT TISSUE INFECTIONS:

Acinetobacter causes about 2.1% of the skin and soft tissue infection in the intensive care unit and it is one of the common pathogen that has been isolated from the burn unit patients which is very difficult to eradicate in these patients. The military people who were injured in Iraq and Afghanistan *Acinetobacter* was the most commonly isolated pathogen it was found to be of low pathogenicity in the skin and soft tissue.

When compared to pneumonia and other infections after the treatment the pathogen was not isolated in repeat cultures from the site and did not contribute to any persistent infection or amputation.

OTHER MANIFESTATIONS:

Acinetobacter is one of the pathogen that causes urinary tract infection it is mostly associated with catheter-associated infections and colonization in ICU- settings and it accounts for about 1.6% of the infection[35].Nosocomial Meningitis is caused by more number of gram negative pathogens which include *Acinetobacter* it is often associated with patients who have surgery and have a external ventricular drain and the mortality in these cases are as high as 70%[36].

Other manifestations include endocarditis involving prosthetic valves, endopthalmitis or keratitis following surgery and a single case of bloody diarrhea.

VIRULENCE POTENTIAL:

Acinetobacter has several factors that contribute to its virulence one important factor is the outer member protein A (Omp) this outer membrane protein binds to the host epithelial cell and mitochondria after which induces mitochondria swelling it is followed by the release of cytochrome c, heme protein that leads to apoptosis[39]. The Omp A is a protein that is found on cell structure that also causes resistance to complement and biofilm formation.

The biofilm produced by the organism play a role in survival inside the host the ability for the bacteria to form a biofilm helps them to grow persistently in unfavorable conditions and environment. The factors that affect its ability to produce biofilm include nutritional availability and presents of pili and outer membrane protein. The initiation of the biofilm formation is done by the pili and a protein called as biofilm associated protein (BAP).

The pili once it gets attached to the abiotic surface it starts the production of microcolonies that develops into a full biofilm structure. The bacterial surface that contain the BAP helps in stabilization and maturation of the biofilm and the environmental signals like cations metals also play role in development of the biofilm formation and adherence of the surface[37].

The other protein that contribute to the virulence factor include phospholipase D and C the phospholipase D causes resistance for human serum epithelial cell invasion, and C causes toxicity in the epithelial cells. Though extensive research is done in the virulence factor only little is known about this emerging pathogen.

MECHANISMS OF RESISTANCE TO SELECTED ANTIBIOTICS

A wide range of antibiotic resistance mechanisms have been described the bacteria *Acinetobacter.* The *Acinetobacter baumannii* has emerged as pathogen that is resistant to beta lactams which also include carbapem it has the ability to respond quickly to environmental pressure.

In France from clinical epidemic samples *A.baumannii* strain, whole genome was sequenced compleatly[38] in which 52 resistant strains have been identified and nearly 45 are located in the AbaR1 resistant island which also includes transposons insertion sequence element and the class 1 integrons.

BETA-LACTAMS:

The resistances to beta-lactams in the bacteria is caused by one of these or combination of these factors like

a) Hydrolysis by beta-lactamases,
b) Alteration in penicillin binding proteins,
c) Change in the structure and number of the porin proteins leading to decrease in antibiotic permeability into the cell and the up regulation of efflux pump that causes decrease in concentration of antibiotics in the bacteria.

CLASS A BETA-LACTAMASES:

A baumannii which belongs to class A extended spectrum beta-lactamases (ESBL's) has the TEM 1 beta-lacmase has be found recently. The strain that has PER-1 display high penicillin resistance and extended spectrum cephalosporins that is ceftazidime minimum inhibitory

concentrations of more than 256µg /ml and cefepime 32 µg/ml but they do not cause resistance to carbapenems. PER-1 is prevalent in Turkey and Korea which has also been reported in this France, Belgium, and Bolivia.

An ESBL *A. Baumannii* which has CTX-M-2 has enhanced ability to hydrolysis cefotaxime and ceftriaxone which is epidemic in countries like Japan and Bolivia[40] but the overall prevalence of CTX –M is low in the *Acinetobacter* sp. When compared to *Enterobacteriaceae*

CLASS B BETA-LACTAMASES:

These beta-lactams are able to hydrolysis betalactam as well as carbapenems with the exception of aztreonam. Class b beta-lactamases have a metallic iron in the active site which is zinc that acts as catalyst which is absent in class A and D Carbapenemases. So they are called as metallo-beta-lactamases. IMP MBL they are found in different parts of the world and they are mostly detected in the class one integron though IMP MBL are not commonly found in *Acinetobacter baumannii*, several types have been described IMP-1, IMP-2, IMP-4, IMP-5, IMP-6 and IMP-11[41].

MBL isolated from Italy a *pseudomonas aeruginosa* encoded by Verona intergron has been reported as VIM-1.*Acinetobacter baumannii* which cointainsVIM-2 has been reported from Korea. The description of Seoul imipenemase SIM-1, a novel MBL, has also been reported, SIM-1 belongs to the B1 sub class. The broad spectrum SIM-1 is 69% homology to IMP-12 MBL and 64% homology IMP-9 MBL it suggests that is gene cassette could have originated from In*55044* superintegron from *Pseudomonas alcaligenes*[43].

CLASS C BETA-LACTAMASES.

In *Acinetobacter baumannii* the bla gene that codes for class C Betalactamases it hydrolysis penicillin and a narrow group of extended spectrum of cephalosporins but cannot hydrolyze cefepime or carbopenams. There are up to 28 variants of bla gene are listed in the gene bank. Like many gram negative bacteria *Acinetobacter* also has chromosomally encoded class C beta- lactamase, ampC gene, it could have been descended from a common beta-lactamase gene ancestry and *Acinetobacter* derived cephalosporinases called as ADC's[44] .

CLASS D BETA-LACTAMASES:

The OXA beta-lactamases belong to the class D which produces oxacillinases and some of the OXA have the ability to hydrolyze extended spectrum cephalosporins but the most important are the OXA which can inactivate even carbapenems. They were first described in a clinical isolate in Scotland OXA – 23 even before the introduction of carbapenems and named as ARI-1 (*Acinetobacter* resistant to imipenem)[45]. Since then it has been discovered in many parts like England, Brazil, Singapore, Korea and China.

An important factor has be implicated to cabapenem resistant in *Acinetobacter baumannii* that they are associated with IS_{Aba1} and ISA_{ba3} which are naturally occurring plasmids that are found in them[47]. These isolates have relatively high MIC for imipenem and Meropenem> 32µg/ml. Most of these oxacillinases are chromosomally mediated. The the carbapenem-hydrolyzing oxacillinases are thought to be chromosomally mediated enzymes plasmid and naturally occurring are given in table 1.

Table 1

OXA carbapenemases in *Acinetobacter baumannii* Carbapenemase type

OXA Carbapenemases[47]

Carbapenemase type	**OXA carbapenemases**
Acquired/Chromosomal	OXA-24, OXA-25, OXA-26, OXA-40, OXA-58*a*
Plasmid	OXA-23, OXA-58*a*
Naturally occurring OXA-51/69 like	OXA-64, OXA-65, OXA-66, OXA-68, OXA-70, OXA-71, OXA-78, OXA-79, OXA-80, OXA-82

An OXA-58 has been described both as chromosomal and as a plasmid-mediated carbapenemase in *A. baumannii*.

OXA-51/69-like betalactamase are located chromosomally in *Acinetobacter baumannii* that has been described in all the parts of the word and called as naturally occurring and their expression depends upon the presents of $IS_{Aba1.}$

CHANGES IN OMPS AND PBPS

In *Acinetobacter baumannii* other non enzynematic mechanisms such as change in outer membrane protein efflux pumps alteration in expression and affinity of penicillin binding proteins also causes both beta-lactam and resistance to carbapenems. The resistance to imipenem and Meropenem has been associated with the loss of a 29-kD protein called as CarO[48] which belongs to the OMP found only in *Moraxellaceae.*

EFFLUX PUMPS:

They are simple and single mechanism that causes resistance to many classes of antibiotics these pumps cause the efflux of compounds that are toxic to the bacterial cell which also includes antibiotics. The efflux pumps that are found in various species of bacteria belong to the small multidrug resistance superfamily the major facilitator superfamily the multidrug and toxic compound extrusion superfamily and the resistance-nodulation –cell division family[49].

The efflux pump that is found in *Acinetobacter baumannii* is called as the AdeABC efflux pump they belong to the family called as the resistance–nodulation-cell division family. These efflux pumps efficiently pump antibiotics like fluro-quinolones, trimethoprim, chloramphenicol,

erythromycin, tetracyclines, cefotaxime and aminoglycosides by over expression of this efflux pump. The expression of these pumps is managed by a two step regulator (*adeR*) and sensor (*adeS*) system. This over expression is due to a single point mutation in the gene *adeR adeS*[50].

AMINOGLYCOSIDES:

The presents of aminoglycosides- modifying enzyme which is coded by the gene present in class 1 integron is prevalent in multidrug resistant *A baumannii.* Recently strain from Japan Korea and united states have described 16S rRNA methlation in *A baumannii (*armA*)* they impairs the binding site and target sites of aminoglycosides to cause high level of resistance to all aminoglycosides which also include amikacin tobramycin and gentamicin.

With the use of PCR mapping it showed that the genes that encode for aminoglycosides modifying enzyme are *aphA1*, *aphA6*, *aacC1*, *aacC2*, *aacA4*, *aadA1*, and *aadB*[51] along with the production of enzymes the AdeABC efflux pump also transport amikacin and kanamycin due to its hydrophilic nature but less effectively when compared to enzyme.

QUINOLONES:

The resistance to quinolones is by modification in the structure of DNA-gyrase which leads to low affinity for the binding of the drug to the DNA complex enzyme. This modification of the structure is caused by mutation[52] in the gene specifically in the region *gyrA* and *parC* they also have efflux pump which includes RND type pump and MATE pump AdeM, so far *qnr* gene which is a plasmid mediated quinolones resistance have not been reported in *A.baumannii.*

TETRACYCLINES:

Efflux pump and ribosomal protection causes resistance to tetracyclines and their derivatives. The efflux pump are coded by *tet(A)-tet(E)* till date the *tet(A)* and *tet (B). tet (A)*[53] coded only for resistance against tetracyclines but has no resistance against minocyclines. Resistance to Tigecycline is conferred by AdeABC efflux pump in *A.baumannii* theses strains show threefold increase in MIC. These efflux pumps are coded by *adeB gene* and these strains are also resistant to gentamicin, levofloxacin, and chloramphenicol.

CARBAPENEMS:

The resistance to carbapenems is caused by the production of beta-lactamses and also contributed to reduced affinity to PBP'S for carbapenems increased efflux of antibiotics decreased permeability of the outer membrane. In the bacteria *Acinetobacter baumannii* the combined action of AmpC betalactamase with weak carbapenemase activity and other may also contribute to it such as decreased production and reduced affinity to PBP's and sometimes loss of OMP's.

Most of the OXA type carbapenemases causes only reduced susceptibility to carbapenems when there are also the presents of secondary mechanism the clinical detection of the organism producing theses enzymes remains to be difficult[54]. The chromosomal location of the gene that encodes contributes to their spread and also due to the increased selective pressure.

The carbapenemases belong to the family of beta-lactamases that has a very broad spectrum of which more efficient than most of the beta-lactam-hydrolyzing enzyme and they are all highly resistant to all commercially found beta-lactamases inhibitors.

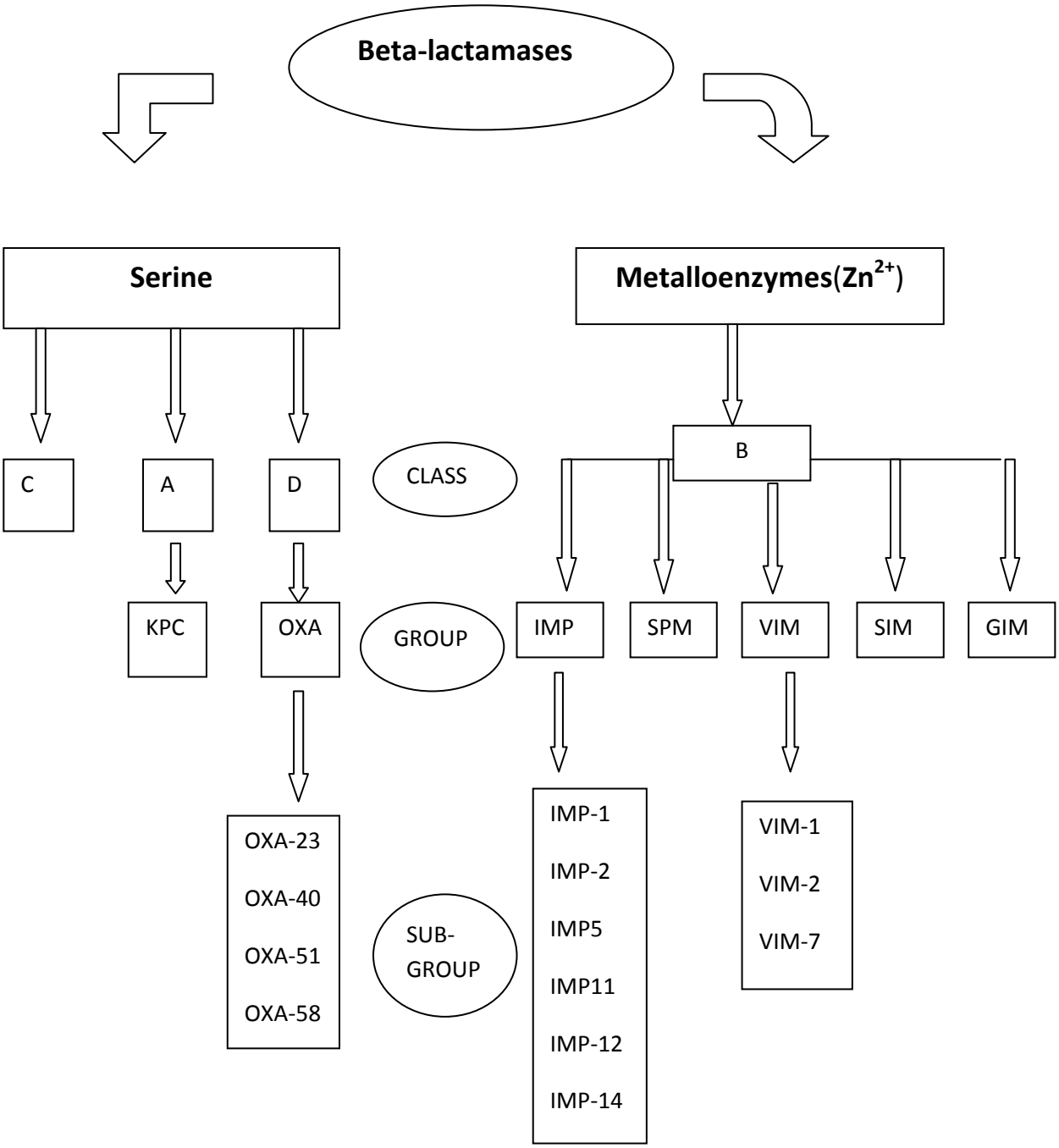
The beta-lactamases are classified on the basis of functional properties and molecular properties

(i) The beta-lactamases were analyzed biochemically and the protein was isolated and by enzymatic studies to determine the hydrolysis and inhibitor profiles were studied.

(ii) It was based on the homology of the amino acid

As a result four major classes where A, C and D which included the beta-lactamases with serine in the active serine and B which had all metalloenzymes with an active site zinc[55].

Classification of carbapenemases into groups and subgroups



MOLECULAR CLASS A CARBAPENEMASES

Class A carbapenems that belong to the functional group 2f, and have been detected in *Enterobacter cloacae*, *Serratia marcescens*, and *Klebsiella* spp. They show reduced susceptibility to imipenem. The class A contains three major families which are NMC/IMI, SME, and KPC enzymes[56] they can hydrolyze a many beta-lactams including aztreonam and carbapems, cephalosporins and penicillins and are all inhibited clavulante and tazobactam.

CLASS B METALLO-_-LACTAMASES

The metallo-beta-lactamases have the ability to hydrolyze carbapenems and they are also resistant to the commonly used beta-lactmase inhibitor but these can only be inhibited using metal ion chelators. The class B carbapenems have to ability to hydrolyze cephalosporins carbapenems and penicillin but they do not have the ability to hydrolyze aztreonam the principle is that the beta-lactams with Zn^{2+} of the active site in the enzyme, which can be chelated only by EDTA or any other divalent cations[51].

The metallo-beta-lactamases are found chromosomally in the prevalent species that are producing these enzyme but the most common families include in it which are VIM, IMP, GIM and SIM are found in

integron structures which becomes incorporated in the plasmids and are readily transferred between bacteria.

CLASS D BETALACTAMASES

This class of betalactamases are also called as oxacillinases or called as OXA type of beta-lactamases when compared to class A and C they all have same serine active sites but differ in amino acid structure. The class B betalactamases are metalo enzymes with Zn ions. Which is not seen in class D several class D beta-lactamases have expanded spectrum of hydrolysis resulting from point mutation. These genes are embedded into class 1 integrons which include insertion sequence and transpoons.

The class D betalactamases are not inhibited by clavulanic acid sulbactam and tazobactum they are also not inhibited by EDTA. The class D betalactamases can be inhibited by NaCl in-vitro this is not seen in other class of beta-lactamases but the property is not clearly understood.

The OXA groups of carbapenems are divided into eight sub groups[54]

- The first group has OXA 23 which is also called as ARI-1(*Acinetobacter* resistant to imipenem) they differ by 2-5 amino acid other enzyme that belong to this group are OXA 27 and OXA 49.

- These vary by 1-5 amino acid in their genetic sequence they contain OXA 24 OXA 25 OXA 26 and OXA 50.

- The third group mainly contains OXA 51 like enzymes and the fourth group only has OXA 58 like cluster which also includes OXA 64-66 OXA 68-71 and OXA 75-78.they diverge by 1-12 amino acid and are naturally occurring in *Acinetobacter baumannii*

- The fifth group have chromosomally encoded enzyme which is seen in Klebsiella pneumoniae which are derived from *shewanella algae* which is chromosomally encoded enzyme OXA -55 and has a enzyme which is derived by five substitution.

- The sixth branch is derived from *Shewannella oneidensis* and differ by 20 substitutions

- The seventh and eight branches of class D are represented by OXA - 50 enzyme of *p. aeruginosa* and the OXA-60 enzymes in *Ralstonia pickettii,* they also have several variants of this enzyme the members of OXA-50 differ with 1-5 substitution while OXA 60 differ by 1-21 amino acid difference.

POLYMYXINS

Polymixin E and polymixin B are used to treat the multidrug resistant *Acinetobacter baumannii* infections polymixin B resistant was reported by Urban et al in 2001 a sub population genetically identical clones which are more resistant than the parent bacteria which was called as heteroresistance have now been described in *Acinetobacter baumannii* the complication of these heteroresistant have to be studied from the patients outcome who are under the treatment of colistin the modification of the lipopolysaccharide that is acylation acidification and the presents of antigen

that interferes with binding of the drug with the cell membrane could be the possible mechanism for the resistance of colistin even though it has not been clear[54].

When compared to carbapenems the resistance to colistin is lower due to their infrequent use as they are reserved drugs used only for the treatment of carbapenem producing bacteria. There has also been treatment failure reports from various parts of the world about 28% of colistin resistant *Acinetobacterbaumannii* reported in Asia.

Hetro-resistance: Among the colistin resistant group of bacteria *Acinetobacterbaumannii* and followed by *K.pneumoniae* and *P.aeruginosa* this resistance occurs due to mutations which lead to bacterial cell membrane changes and specific outer membrane protein content and cross resistance that occur between colistin and polymixin B[59].

The colistin-susceptible strains that harbouring a resistan sub-population this is called as heteroresistance but the impact of this heteroresistance clinically and anitibiotic treatment strategies have yet to be seen. This can be prevented by using colistin as a part of combination therapy and also by drug interval duration and dose.

MONOTHERAPY:

In the resent studies it has come to light that decrease in concentration of the drug there is re-growth of the bacteria that survive. This decrease in concentration is due to[59.]

- The majority of the drug is excreated in the urine before it is converted into its active form.
- The drug binds with α1-acid glycoprotein an acute phase protein that is found in critically ill patients that combines with the drug to inactivate it.

These facts suggest that the colistin should be given as a part of combination to act synergistically against the pathogen and decrease the enzyme resistance. Now there is a compelling need in microbiology to perform the synergy testing methods to determine the synergistic combination in vitro and vivo.

OTHER ANTIBIOTICS

The resistance to trimethoprime-sulfamethoxazole and chloramphenicol resistance have also been reported in multidrug resistant *Acinetobacter baumannii* strains. The gene encoding for the resistance for these drugs (*dhfr &cat*) have been found inside the 3´ conserved region of the integron which also has *qac* gene that confrere to resistance to antiseptics and sulfonamides[60]. The role of efflux pumps in the resistance against these antibiotics also could be possible.

The presents of an intrinsic chromosomal cephalosporinase (AmpC) is the most common mechanism of resistance in *Acinetobacter baumannii.*[62]

MECHANISMS OF ANTIBIOTIC RESISTANCE IN *ACINETOBACTER* SPECIES [62].

Mechanism	***Acinetobacter* species**
β-Lactamases	
AmpC cephalosporinase	+
Inducible	–
TEM	+
SHV	+
CTX-M	+
PER	+
VEB	+
OXAa	+
IMP	+
VIM	+
SPM	–
GIM	–
PSE	–
GES	–

Mechanism	***Acinetobacter* species**
IBC	–
OMP changes	+
AMEs	
Adenylating	+
Phosphorylating	+
Acetylating	+
Topoisomerase mutations	
gyrA	+
parC	+
Efflux pumps	+
Mobile genetic elements	+
Integrons	+
Membrane changes and resistance to polymyxin	–

MANAGEMENT OF *ACINETOBACTER BAUMANNII:*

Acinetobacter may colonize the skin pharynx GIT urethra conjunctiva and vagina so care must be taken to rule out colonization and environmental contamination. The colonization does not require any special treatment but the identification of resistance to multiple antibiotics are of at most important.

For the susceptible strains they have been treated with beta-lactams mostly the third generation and extended spectrum penicillins have been used for the treatment. In the case of severe infection penicillin with beta-lactam inhibitors and cabapenem with combination with aminoglycosides have been used[63].

In the management of *Acinenatobacter baumannii* and *Acinetobacter* spp the CLSI recommends broth dilution or disk diffusion test these test are performed using Muller-Hinton agar or cation-adjusted Muller-Hinton broth direct colony suspension, equivalent to 0.5 McFarland standard Incubation at 35°C. They are tested against the following groups of drugs, penicillins, betalactamase inhibitor combinations, cephems (including cephalosporins I, II, III and IV), carbapenems lipopeptides,

Aninoglycosides, tetracycline fluoroquinolones and folate pathway inhibitors.

GLOBAL EPIDEMIOLOGY

Acinetobacter baumannii is now an emerging carbapenem resistant bacteria worldwide it has been shown that the percentage of carbapenems resistant bacteria have increased over the last ten years like North America, Europe and Latin America. In Europe they have been reported in northern parts united kingdom, Spain, Portugal, France, Poland, Netherlands and Czech republic the countries from southern Europe and middle east include Turkey Italy, Greece, Bulgaria, United Arab Emirates, Iran, Iraq and Israel.

A.baumannii is not confined to one hospital in the city it can occur national wide like the spread of the OXA23 clone to England the disseminated of multidrug resistant clone in Portugal. Intercontinental and international spread has also been identified between Europe and other places as a result of airline travel[64]. These events show the importance of screening and isolation of the patients being transferred from countries with high prevalence of drug resistant organism.

According to the Antimicrobial SURVEILLANCE REPORT resistance surveillance in Europe 2012 more than half of the isolates of *Acinetobacter baumannii* resistance to carbapenems were high along with most cases of combined resistance to other antimicrobial groups the counties like Iceland, Luxembourg and Malta reported less than 10 resistant isolates. The lowest was reported from Netherlands 1.7% and the highest was seen in Italy 83.1% for aminoglycosides. More than 18 countries reported for Fluoroquinolones the least no isolates were from Norway and Netherlands and the maximum was seen in Greece93.1%.

For Carbapenems in the year 2012 overall 3190 were reported in Europe the least was reported from Norway >1 to 87.8% in Italy. The combination resistance was seen more common over all 4.9% was single drug resistance and 17% reported for two classes of drugs, the most common resistance was seen to fluroquinolones and combination with aminoglycosides or to carbapenems or both were more common.

In north America after an out-break of ESBL producing *Klebsiella* for which imipenem was significantly used, following which the infection due to carbapenems resistant *A baumannii* was reported in large number of hospitals[65] in 1991 and 1992 After which numerous out breaks have been reported of multi drug resistant *A baumannii.*

In the United States most common bacteria isolated from gram-negative bacilli active surveillance cultures, 1 June 2009–31 May, 2012 *Acinetobacter baumannii* was one was the most common isolated bacteria only next to *Escherichia coli, Klebsiella pneumoniae* and *Enterobacter aerogenes*[66]. In which 44.8% of the isolates were multidrug resistance isolates, 33.6% were aminoglycosides 36.4% were betalactamases 44.1% were carbapenems and floroquinolones.

In the Middle East[67] the most commonly isolated gram negative non fermentative organism was *P. aeruginosa* (72.9%), followed by *A. baumannii* (25.3%) and *S. maltophilia* (1.8%) was reported in March 2012. It was found that of the total number of the isolated *Acinetobacter baumannii* >50% of the isolates were resistant strains which showed about 76.9% resistance to amikacin, 77.8% resistance to gentamicin, 5.4 %

resistance to Imipenem, 95.5% to aztreonam, 90.6% to tetracycline 95.9% to chloramphenicol, and 13.2% resistance to polymyxin-B.

In India a study done over a period of one year from January to December 2013 from various tertiary care hospitals in Delhi and other parts of India was done which reviled large number of isolates that were multi drug resistant[68]. Of the total number of isolates 88.60% showed resistance to Cefotaxime 88.94% to amikacin 80.54% to gentamicin moderate number of isolates were resistant to Piperacillin tazobactam 32.98% and also with netilmycin which was 39.04% and low number of isolates was found to be resistant to Imipenem 15.22% meropenum was found to be 7.32% and less than 2% for colistin and polymyxin B.

In a study conducted in 2010 by AIIMS it was found that about 17.32% of isolates was resistant to Imipenem and in Vellore by Anitha et al resistance to carbapenems was reported to be 96.6% and in Mumbai that showed 29% was resistant to Imipenem.

GLOBAL EPIDEMIOLOGY OF CARBAPENEM RESISTANCE IN ACINETOBACTER BAUMANNII;

The exposure of *Acinetobacter baumannii* to various groups of drugs in the intensive care units gradually, due to selective pressure has led to the prevalence of resistant to carbapenems worldwide. The relevant carbapenamases gene in *Acinetobacter baumannii* such as cephalosposporianases and oxacillinases have all been described in the bacteria[71]

Table 2 Genes of carbapenemase production.

Enzyme	Described in association with	Isolated in
IMP-1	Class 1 integron	Italy, Japan, India N.Korea
IMP-2	Class 1 integron	Italy
IMP-4	Class 1 integron	Singapore, Hong Kong
IMP-5	Class 1 integron	Portugal
VIM-2	Class 1 integron	North Korea
VIM-1	Class 1 integron	Greece
VIM-3	Class 1 integron	Taiwan
VIM-4	Class 1 integron	Greece
SIM-1	Class 1 integron	China, South Korea

Enzyme	Described in association with	Isolated in
NDM-1	Chromosomal/ISAbal 25; plasmid	India Germany, china Bangladesh, Belgium.
NDM-2	chromosome	Egypt Israel arab emirates
OXA-23	Chromosome/plasmid	UK,USA, France, brazil Italy Greece
OXA-24/40	Chromosome/plasmid	Portugal, Spain, Italy, Greece, UK, USA Spain
OXA 58	Chromosomal/plasmid	France, Spain, Italy, Greece, UK, Austria, Romania, Iraq, Kuwait, Lebanon, Turkey

The OXA 51- like enzyme cluster vary from other OXA type of carbapenemases as it is chromosomally located also inherent in *Acinetobacter baumannii*[55] but its activity depends on the presence the IS element. The most commonly found type of OXA carbapenemase which is $bla_{oxa\text{-}24\text{-}like}$ which similar to OXA-23 like enzyme this gene was located on plasmid. The $bla_{OXA\text{-}58\text{-}like}$ is a plasmid mediated and has been reported in many hospitals out breaks and found with the association of ISAba2, IS18

element in all strain which suggested that this might been acquired by horizontal transfer of gene.

Combination therapy.

The combination therapy for the treatment of gram negative bacteria was due to the following reasons

- Delay or prevent the occurrence of resistance in the bacterial strain due to antibiotic therapy[70].

- To improve the clinical outcome for the patient by using the synergistic effect.

- To increase the impirical coverage that is provided by the different spectrum of activity of the drugs.

Even though there is a strong appeal for this approach there are only few strong evidence to support for the treatment of drug resistant gram negative infections and the addition of the second drug also cause increase in drug resistance increase in costs and adverse effect like for example nephrotoxicity related to aminoglycosides all have be well noted

There are some studies that have shown decrease in mortality in patients who have treated with combination therapy in a retrospective study conducted by in 281 intensive care units between combination therapy with betalactams along with aminoglycosides /macrolites /fluroquinolones compared with betalactam monotheraphy which resulted in decreased mortality of 36% and 29%. When a combination therapy can be prescribed the second antimicrobial agent that has been chosen should have activity against the organism which is potentially resistant to the beta-lactam. The selection should be based upon[70]

- Use of epidemiology of the local susceptibility pattern.

Individualization of combination therapy based on patient characteristics because as patients differ with regard to preexisting medical conditions, severity of illness, nature of infection, previous antibiotic and hospital exposure, presence of indwelling catheters, and colonization with antibiotic-resistant organisms.

Methods to test for antibiotic drug synergy

There are many methods use in-vitro to test the synergy, the methods described are modifications of the E-test, checkerboard assay and time-kill assay.

Time-kill assay:

Here the bacteria is allowed to grow in a liquid medium in which the test antimicrobial agent is incorporated and the viability of the bacteria is checked at different interval the test is done by the addition of the second drug intended to be used in the combination therapy and other with a single drug[71]. At defined time intervals specific amount of broth is sub-cultured on to the agar medium and the number of colonies are counted. It is found to synergy if there is decrease in 2 $\log_{10}$ between drug combination and the single drug.

Checkerboard assay:

Here in a 96 well micro-plate the mic the MIC if test drug A the MIC of test drug B and MIC of the combination (A+B) are determined the total fractional inhibitory concentration (ΣFIC) is calculated which is the interaction between drugs A and B ΣFIC = FIC of drug-A + FIC of drug-B; where FIC of drug-A = MIC of drug A / MIC of (A + B) combination and FIC of drug-B = MIC of drug B / MIC of (A + B). The ΣFIC values ≤ 0.5 is interpreted as synergy, 0.5 - 4 as indifference and the values > 4 indicate antagonism[72].

E-test methods

Drug synergy detection in combination using Epsilometer strips can be done by three different methods[73]

1. Fixed ratio method: in this method the test organism is streaked in a Petri dish and the e strip of drug a is placed in it and the MIC are marked using a sterile needle then incubated for an hour then the e strip A is replaced by the E strip containing the B drug such a way that the MIC of drug A and B coincide with each other then the plate is incubated for 18 hours and readings are taken.

2. The second method is done by placing the two E strips at right angle to one another at the same time in such a way that the MIC intersect with each other and the readings are taken after 18-24 hours like the previous method.

3. The third method: in this method one of the drugs is dissolved in t media (most potent drug) in a fixed concentration and the E strip for the second drug is placed over it.

In all the three methods the results can be calculated similar to Checkerboard assay by calculating the ΣFIC value.

Different Synergy Testing Method's Performance:

The time kill assay method is considered to be the better among the in-vitro methods to test for synergy testing and is considered to be the gold standard. The E test methods are considered to be the most easy to perform and also the results can be provided earlier and the third method in which the drug is incorporated in the media has proved to show correlation with time kill assay but the Epsilometer strips are expensive to be used in routine diagnostic work. The checker board assay provides reliable results, the checkerboard assay and time-kill assay both faces the disadvantage of being too labor intensive for routine diagnostic work. The advantage over time kill assay is that can detect antagonism better than tike kill assay and some studies have shown good correlation with clinical outcome[74].

Animal models:

Animal model serves as a guide to test the efficacy in vitro combinations. In the animal model the results of the in vitro test have be contrast to the in-vivo test, in the guinea pig model tested for *Acinetobacter baumanni* Imipenem sensitive strains it was found to be less effective when

it was treated with Imipenem and amikacin than in mono therapy of the same antibiotics. Similarly the combination of Imipenem with levofloxacin combination did not achieve bactericidal activity in animal model. Similarly it was seen in the combination of amikacin and doxycycline[75].

The combination of rifampin with Imipenem tobramycin or colistin demonstrated in mouse pneumonia models with Imipenem resistant strains showed good results when compared to Imipenem when compared to Sulbactum. In an inter-peritoneal infection high survival rate was observed in the combination of meropenem and Sulbactum than in monotherapy of both the drugs[76].

Rifampin had shown good efficacy in different animal models with various drug combinations and the results of colistin with combination of other drugs was not as effective in both pneumonia and endocarditids models[77].

Clinical studies:

During the past two decade treatment of these multi-drug resistant *Acinetobacter baumannii* have proven to be difficult and there is still no prospective studies to guide for the combination treatment for this

organism, on a study done with critically ill patients only four patients showed improvent with the combination of carbapenem and Sulbactum and the study concluded as Sulbactum could provide an alternative in cases where tigecycline and colistin are not available[78].

For the treatment of meningitis caused by multidrug resistant *Acinetobacter baumannii,* combination of amikacin and colistin and concluded that lack of newer agents could lead to difficulty in treatment if resistance may develops for combination of drugs also[79]. Though several articles has shown positive results with rifampin and Imipenem combination there also some studies that has shown negative results with the same combination.

4. MATERIALS & METHODS

MATERIALS AND METHODS

STUDY PERIOD: April 2013 to July 2014.

STUDY TYPE: Prospective and descriptive study.

SAMPLE SIZE: 77

$$n=4pq/d^2$$

n = required sample size

p=expected prevalence

q=100-p

d= degree of prevalence

$$n = 4 \times 5.1 \times (100-5.1) / 5^2$$

$$=77.43$$

STUDY POPUPLATION:

Among the in clinical isolates obtained from both the in-patients and out-patients in out hospital who visited our hospital during the duration of the study.

Inclusion criteria:

All multi and pan drug resistant *Acinetobater* sp isolates were included in the study.

Exclusion criteria:

All susceptible strains of *Acinetobacter* sp were not included.

Ethics approval:

Approval was obtained from the Institutional Ethics committee before the start of the study. It was renewed during the period of the study.

STUDY DESIGN:

WORK FLOW DIAGRAM

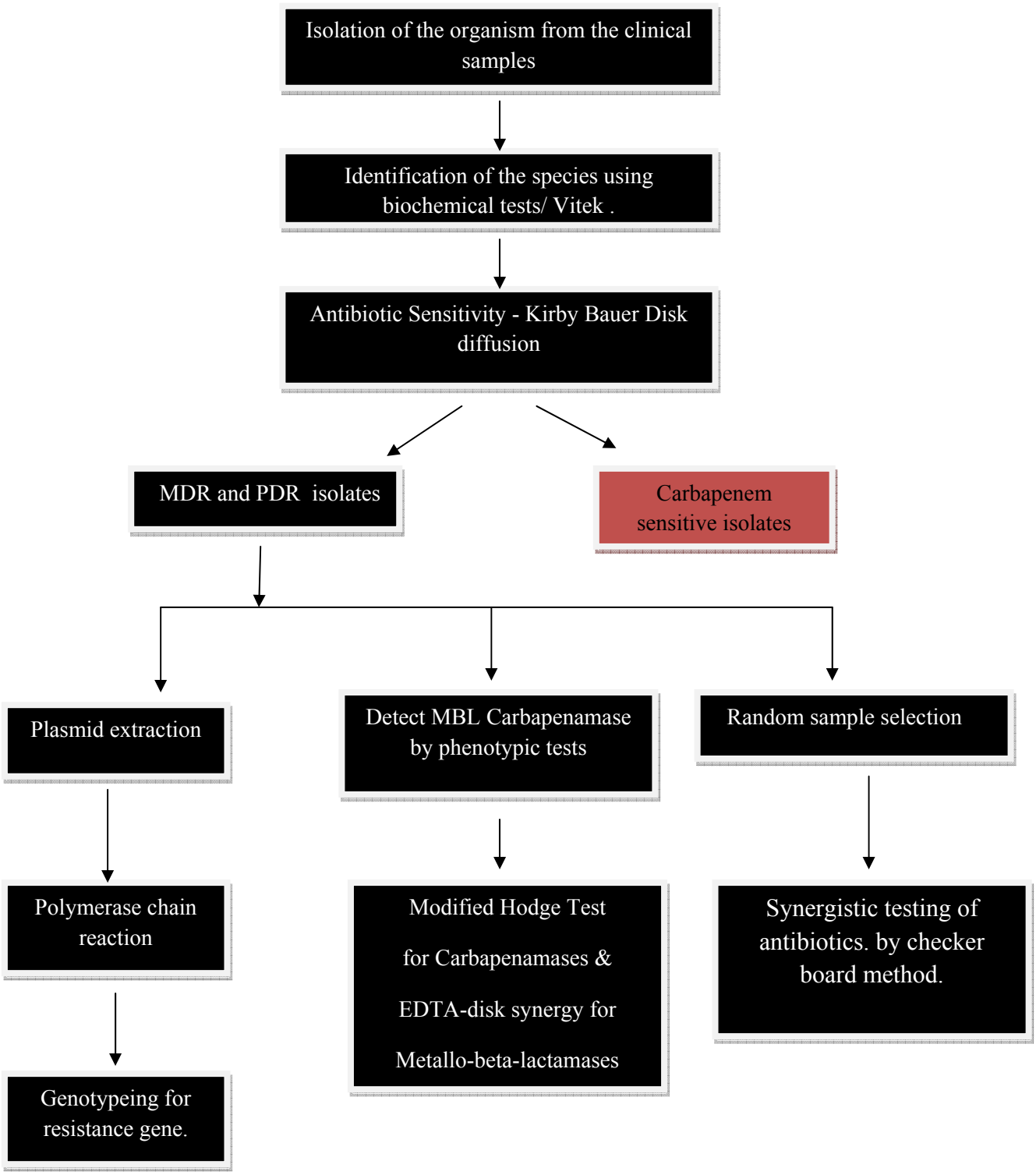
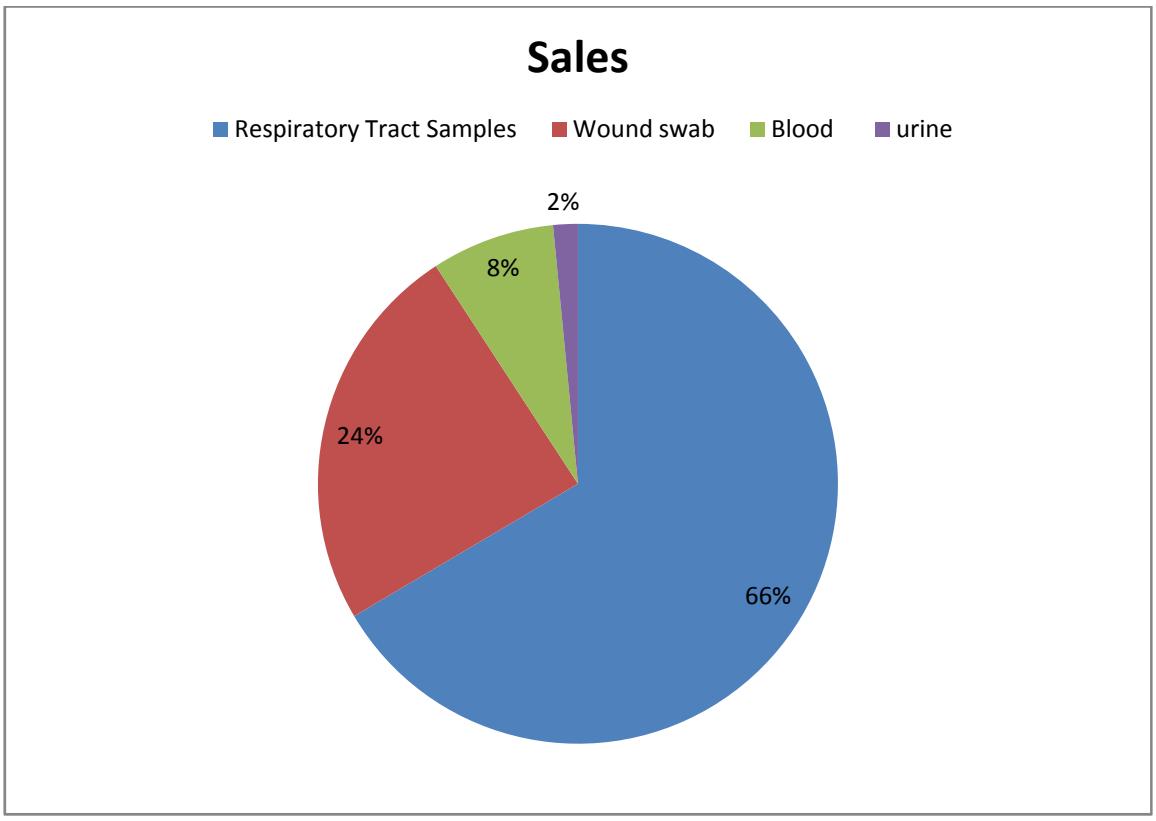


Figure 1

DISTRIBUTION OF BACTERIAL ISOLATES IN VARIOUS SAMPLES



Identification of the bacteria by phenotypic methods:

The isolates from the clinical sample underwent various phenotypic test based on the method described be Gerner smidt the isolates. In the isolates the test like gram stain, oxidase test, motility and growth in blood agar and Mac Conkey Agar was done and the organism which was gram negative oxidase negative and non motile underwent other test for

identification which in brief included 1% glucose containing media in Hugh and leifson's medium, gelatin liquefaction and assimilation tests which contained 0.1% t-Aconitate, $_{L}$-histidine, malonate and citrate also susceptibility to penicillin G (10µg) and chloramphenicol (30µg).

The isolates were also identified using VITEK automated microbial identification system. It was done by isolating a pure culture and suspending 2-3 colonies in saline, it is to be prepared to match 0.5 Mc Farland standard and using the identification for gram negative bacteria card GN (gram negative) they were identified.

Based on the phenotypic test done the *Acinenatobacter* species were differentiated, based on the table.

Table 3 Phenotypic characters of *Acinetobacter* species.

Susceptibility to Chloramphenicol		3	19	48	65	100	5
Susceptibility to Penecllin		3	0	67	70	97	32
Citrate		100	76	86	90	11	0
Malonate		86	0	0	20	0	95
l- histidine		97	0	0	0	20	0
t-Aconitate		84	76	0	0	4	0
Growth at 37°	44°c	51	0	0	0	0	0
	37°c	97	100	100	0	76	100
Haemolysis		0	95	0	0	0	0
Gelatin liquefaction		0	90	0	0	0	0
Glucose oxidation		99	76	0	0		5
Organism		*Acb-complex*	*A. haemoliticus*	*A. junii*	*A. johnsonii*	*AA. loffii*	*A. radioresistens*

ANTIBIOTIC SUSCEPTIBILITY TESTING:

The antimicrobial susceptibility tests for the *Acinetobacter* sp was done by Kirby-Bauer disc diffusion method as per the Clinical and Laboratory Standards Institute (CLSI-2013) guidelines using Muller- Hinton (MH) agar. The results were also interpreted as per the same guidelines. The following drugs were all tested and they were also tested by vitek automated system with the card AST-N281 Gram negative card susceptibility card to which 145µl of 0.5 Macfarland standard of the coloney suspention is added to 3 ml of the saline. Then by broth dilution in the machine the susceptibility was found.

Table 4 Break points for various antibiotics.

Antimicrobial agent (µg)	**Zone size (mm)**
Piperacillin 100	>21
Ticarcillin 75	>20
Ampicillin-sulbactam 10	>15
Piperacillin-tazobactam 100	>21

Antimicrobial agent (µg)	**Zone size (mm)**
Ticarcillin-clavulani acid 75	>20
Ceftazidime 30	>18
Cefepime 30	>18
Ceftriaxone 30	>21
Imipenem 10	>16
Meropenem 10	>16
Polymyxin B	MIC<2
Colistin	MIC <2
Gentamicin 10	>15
Amikacin 30	>17
Tetracycline 30	>15
Doxycycline 30	>13
Ciprofloxacin 5	>21
Levofloxacin 5	>17
Trimethoprim-sulfamethoxazole	>16

Screening test for carbapenemase detection:

- The isolates that produce carbapenemase are found to be resistant to carbapenems and also to other beta lactum antibiotics.

- The carbapenemase producing isolates show intermediate or resistance to one more carbapenems and also test resistant to one or more agents of class III (ceftriaxone Ceftazidime cefoperazone Cefotaxime)

- In *Acinetobacter* sp, It can also be identified by the performing broth dilution which have Imipenem >8 µg /ml.

CONFIRMATORY TEST FOR CARBAPENEMASES.

As per CLSI the confirmatory test which is done for the isolates which produces carbapenemase is Modified Hodge Test. It is based on the principle that the carbapenemase enzyme produced by the strain will diffuse into the culture media and the sensitive indicator strain will still grow even in the presents of Imipenem near the strain producing an indentation.

Procedure:

The ATCC *E coli* strain was inoculated on the surface of Mueller Hinton plate from a fresh preparation of 0.5 Mc Farland suspension.

In the center of the inoculated plate Meropenem (10µg) disc were placed then the test organism is streaked from the edge of the disc in a straight line. The plates were then incubated in 37°C for 16-24 hours

Interpretation

The plate was then examined after incubation for the clover leaf pattern at the point of intersection of the test strain and the ATCC strain, the results were interpreted as positive or negative.

If the ATCC organism has a clover leaf like indentation along the growth of the test organism in the disc diffusion zone, then called as Modified Hodge Test positive.

DETECTION OF CLASS A CARBAPENEMASE

Boronic acid inhibition test:

The test_organism was prepared to 0.5 Mc Farland suspension and lawn culture was inoculated on MHA plate, then either of Imipenem/ meropenem/ ertapenem disc and another disc was placed along with it 400 µg phenylboronic acid in the inoculated Muller Hinton agar plate.

The plates are incubate at 37°C for 18-24 hours

Interpretation

Of the both antibiotic tested in combination with phyneyboronic acid the zone difference is greater than 5 it was interpreted as positive for the production of class A carbapenemase.

DETECTION OF CLASS B CARBAPENEMASE

Combined disk test (EDTA disk enhanced test).

Principle: in the presents of Ethylenediaminetetraacetic acid (EDTA) the metallo beta-lactamase enzyme is inhibited.

Prepatration of EDTA stock solution

EDTA 0.5M solution was prepared by dissolving 186.1 g of disodium EEDTA.2H_2O in 1000ml of distilled water and pH of 8.0 and it was sterilized by autoclaving

The test strain suspension was made with 0.5 Mc Farland and it is spread over a Mueller Hinton Agar plate then two antibiotic disc one was Imipenem (10μg) and the other Imipenem with EDTA added to the disc.

Then the plates are incubated at 35°C for 16 to 20 hours.

Interpretation

A zone size of greater than 5 mm between the two disc (EDTA combination > than the Imipenem disk) is conformation for the production of MBL production**.**

DNA EXTRACTION FOR POLYMERASE CHAIN REACTION (PCR)

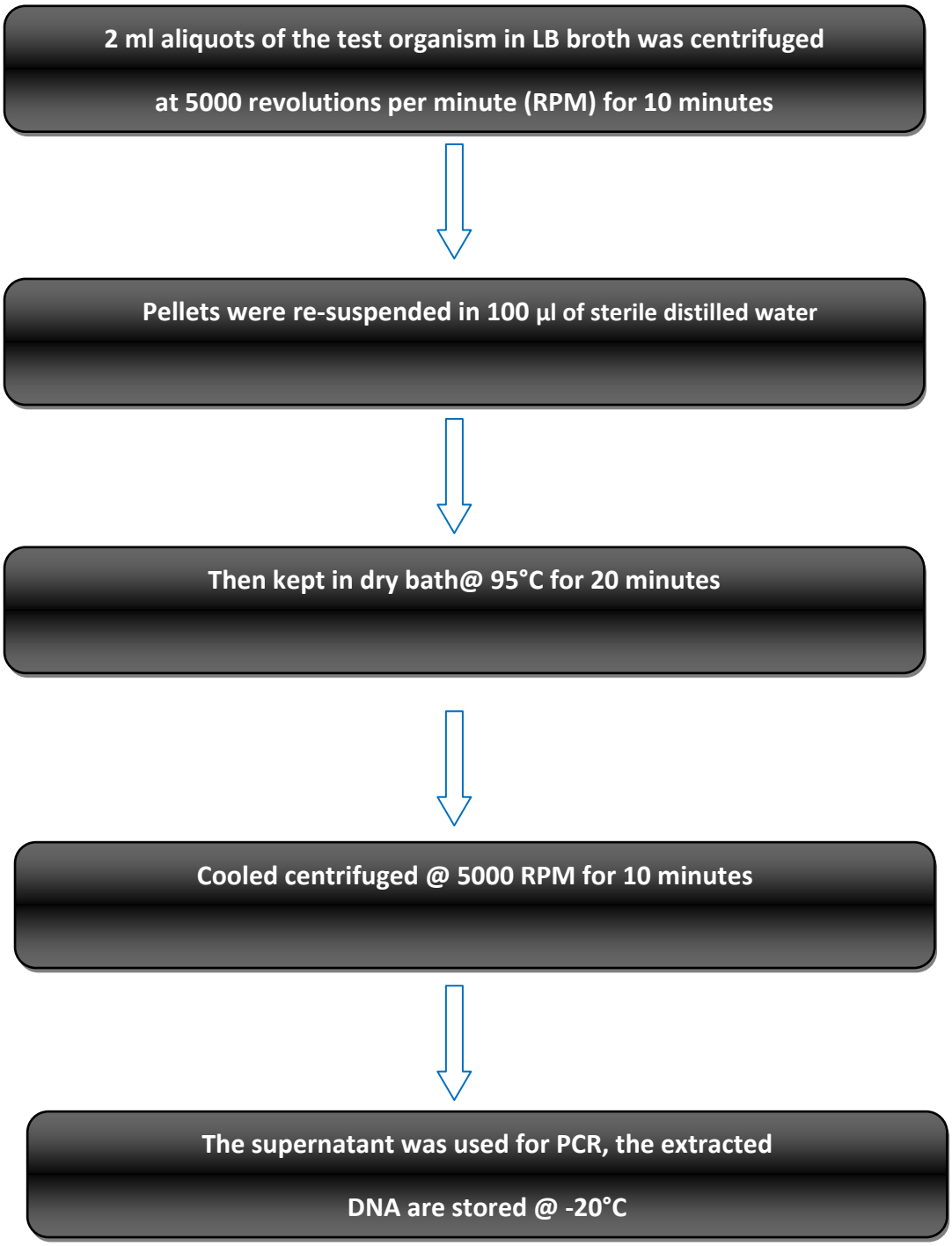
Preparation of isolates

The isolates from which the DNA where extracted was first inoculated in Luria-Bertani medium (broth) which contained for 1000 ml of distilled water 5 grams of sodium chloride 5grams of yeast extract and 10 grams of trypton. The broth was incubated overnight and two to three ml was used for extraction

Table 5 Composition of Luria Bertani broth.

S. No	INGREDIENTS	QUANTITY
1.	Tryptone 1.0% [w/v]	10 g
2.	Yeast extract 0.5% [w/v]	5 g
3.	NaCl 0.5% [w/v]	5 g
4.	Water	1000 ml

DNA Extraction Procedure



PCR for the detection of OXA Genes:

PCR was performed for 61 the isolates which are phenotypically confirmed to express c carbapenemase.

The primers used were obtained from Sigma Aldrich, Mumbai, which were based on reference article from a previous study. The primers used were tabulated below

Table 6 Primers used for the PCR extraction.

S.no	Name	Sequence(5′-3′)	Amplicon size (bp)
1	OXA-51-like	F- TAA TGC TTT GAT CGG CCT TG R- TGG ATT GCA CTT CAT CTT GG	353
2	OXA-23	F- GAT CGG ATT GGA GAA CCA GA R- ATT TCT GAC CGC ATT TCC AT	501
3	OXA-24	F- GGT TAG TTG GCC CCC TTA AA R- AGT TGA GCG AAA GGA TT	246
4	OXA-58	F- AAG TAT TGG GGC TTG TGT TG R- CCC CTC TGC GCT CTA CAT	599

Reaction mixture:

Each single reaction mixture (25µl) contained 5 µl of DNA suspension, 15pmol of each primer (Sigma- Aldrich, Mumbai), 10mM dNTPs, 1 U *Taq* DNA polymerase, 25mM $MgCl_2$ and 2.5 µl of **10** x *Taq* buffer.

POLYMERASE CHAIN REACTION:

The PCR was performed by conventional method using a thero-cyler, under the following conditions, which were same for all the four genes except the Annealing temperature alone was different for each of the different set of primers that are given in the table below.

Initial denaturation at 94°C for 5 minutes, 30 cycles of 94°C for 25 seconds, Annealing temperature based on the gene which is amplified and extension at 72°C for 50 seconds, and a final elongation at 72°C for 6 minutes

Table 7 Temperature used in thermocycler for PCR amplification.

NAME	Denaturation temperature	**Annealing temperature**	Extension temperature
OXA-51-like	94°C	51°C	72°C
OXA-23	94°C	58°C	72°C
OXA-24	94°C	58°C	72°C
OXA-58	94°C	59°C	72°C

The products after amplification are stored at **-20°C** until they were subjected to agarose gel electrophoresis.

AGAROSE GEL ELECTROPHORESIS:

The amplified product are visualized by use of agrose gel electrophoresis, the agrose gel was prepared to a ratio of 1% by adding agrose gel powder with tris borate EDTA (TBE) buffer. This solution was heated in microwave till it becomes a clear solution and followed by the

addition of Ethidium bromide to visualize the amplified DNA under UV light.

A 100 base pair ladder was used as the molecular marked to measure the amplified product from the PCR in the first well and the rest of the wells had 5µl of the amplified product along with 3 µl of the loading dye from Sigma Aldrich was used to visually see the movement of the product. The gel electrophoresis was performed by placing the gel in an electrophoresis tank containing tris borate EDTA (TBE) buffer at 74 volts for 45 minutes.

Interpretation:

The ethidium bromide was used to stain the amplified DNA for visualization under UV illumination. Then images of the gel are captured by Gel doc. The presence of the amplified DNA fragments of the OXA genes is confirmed by the formation of bands corresponding to their molecular weight and a 100bp ladder that was used for this purpose.

DRUG SYNERGY TESTING

Checkerboard method:

A standard 96 well plate was used to perform the drug synergy testing and dugs Rifampin Amikacin Colistin and Ciprofloxacin from Sigma Chemical Co and Imipenem from GlaxoSmithKline (GSK) and sulbactam from was used they were stored in 2- 8°C until use.

The drug solution was prepared by using the appropriate solvent and solution as per CLSI guidelines before the test was performed

Table 8 Solvents and Solutions used for Antibiotics.

Antibiotics	Solvent	Solution
Amikacin	Water	Water
Ciprofloxacin	Water	Water
Colistin	Water	Water
Imipenem	Phosphate buffer solution pH 7.2,0.01 mol/L	Phosphate buffer solution pH 7.2,0.01 mol/L
Rifampin	Methanol	0.1 M phosphate buffer ph 7.4 +0.45%sodiu dodecyl sulfonate
Sulbactam	Water	Water

The drugs were taken and dissolved and diluted in the appropriate solution and further diluted by doubling dilution method.The fresh grown isolate of *Acinetobacter baumannii* was taken from MHA and using direct colony suspension was taken in saline and a standard suspension of 0.5 Mcfarland standard is prepared, then in double strength Mueller Hinton broth 25 times so the number of bacteria equal to ~3 X 10^5CFU/ml

Method

1. Except the row 1, in each well 50µl drug A is added to each well from higher to lower concentration.

2. Except the column 1 in each well 50µl of drug B is added from higher to lower concentration.

Table 9 Arrangement of antibiotic concentration in checker board.

(A1)	Drug A 64mg/ml	Drug A 32 mg/ml	Drug A 16mg/ml	Drug A 8 mg/ml
Drug B 2mg/ml				
Drug B 1mg/ml				
Drug B 0.5mg/ml				

Then 100µl of the inoculum is added to each well and the control well A1 (no drug)

Then the plate is gently shaken to mix the drug and inoculum and the plate is kept for incubation

Interpretation:

After twenty four hours of incubation at 37°C the results are read visually by looking for turbidity if the growth of the micro-organism in the plate and the results are noted.

The MIC of the combination (A+B) are determined the total fractional inhibitory concentration (ΣFIC) is calculated which is the interaction between drugs A and B ΣFIC = FIC of drug-A + FIC of drug-B; where FIC of drug-A = MIC of drug A / MIC of (A + B) combination and FIC of drug-B = MIC of drug B / MIC of (B + A). The ΣFIC values ≤ 0.5 is interpreted as synergy, 0.5 - 4 as indifference and the values > 4 indicate antagonism.

Table 10 model of micro-titer plate used for drug synergy testing.

		Drug B			
Drug A	**control**	64 mg	32 mg	16 mg	8 mg
	2 mg	No growth	No growth	No growth	No growth
	1 mg	No growth	No growth	growth	Growth
	0.5	No growth	growth	growth	Growth

MIC of drug A(colistin) = 64

MIC of drug B (imipenem or rifampin or ciprofloxin or sulbactum) = 2

MIC of drug A+B = 0.5

MIC of drug B+A =32

5. RESULTS

RESULTS

During the study period **6.8%** of the total positive sample belonged *Acinetobacter* sp in our lab, which was next only to *Escherichia coli* (64.89%)*Pseudomonas aeruginosa(9.8) candida(9.6) kiebsiella pneumoniae(8.6) and Staphyloccus aureus(7.6)* in our lab. Which were identified based on colony morphology pale lactose fermenting colony on MacConkey agar gram negative oxidase negative nonmotile bacteria figure shown below .

Figure 2 growth of *Acinetobacter* on Mac-agar.

The total number of *Acinetobacter* sp which was isolated in the lab was found to be 162

The prevalence of each species of *Acinetobacter* based on the species were as follows

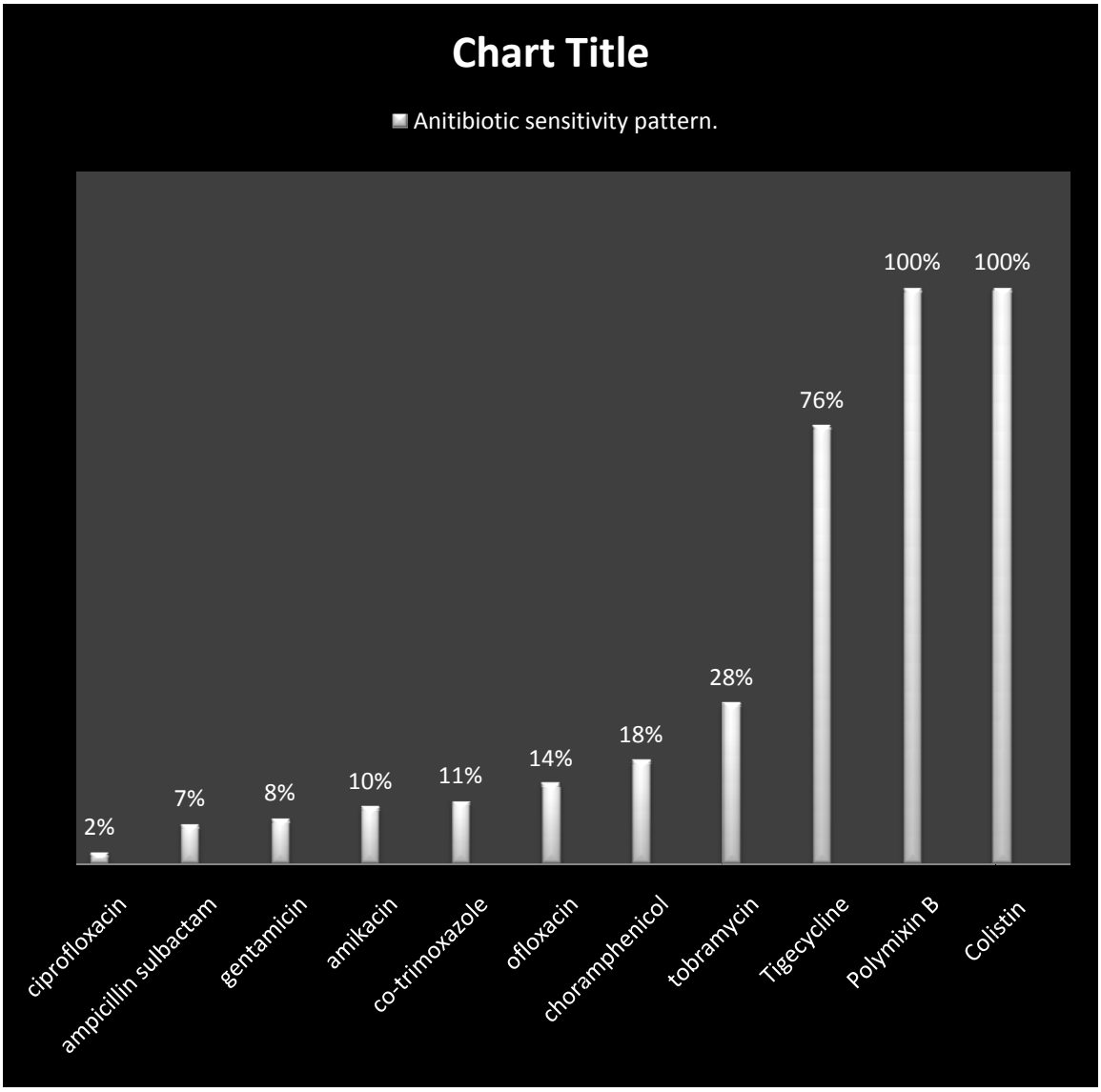
Table 11 Distribution of various samples from which *Acinetobacter* was isolated.

Organism	**Number**
Acinetobacter baumannii	145
Acinetobacter species	13
Acinetobacter loffii	3
Acinetobacter haemolyticus	2
Total	**162**

Of total number of *Acinetobacter* Sp 58.02% were carbapenem resistant strains and they all belonged to *Acinetobacter baumannii.*

Antibiotic Susceptible of the carbapenemase strain are shown in the figure 2.

Figure 2 Antibiotic susceptibility pattern.



Of the carbapenems resistant strain obtained it was found that 73% if the isolates were multi drug resistant and were only sensitive to colistin and one other group of drug (Tigecycline or trimethprime/sulfamethoxazole) and only 2 isolates were resistant to all drugs pan drug resistant.

For all the strains that were suspected to screening test (Modified Hodge Test) was done and then confirmatory test was also done for strains of the total 162 samples 94 samples were carbapenemase producing strains the Modified Hodge was done.

In the 94(resistant to carbapenems group of drugs) strains which tested for carbapenemase production 100% were positive by Modified Hodge test and only 18.08% were positive by EDTA disk enhanced test(for group A) and only 2.12% by boronic acid test.

Figure 3 Phenotypic identification of cabapenemase producting isolates.

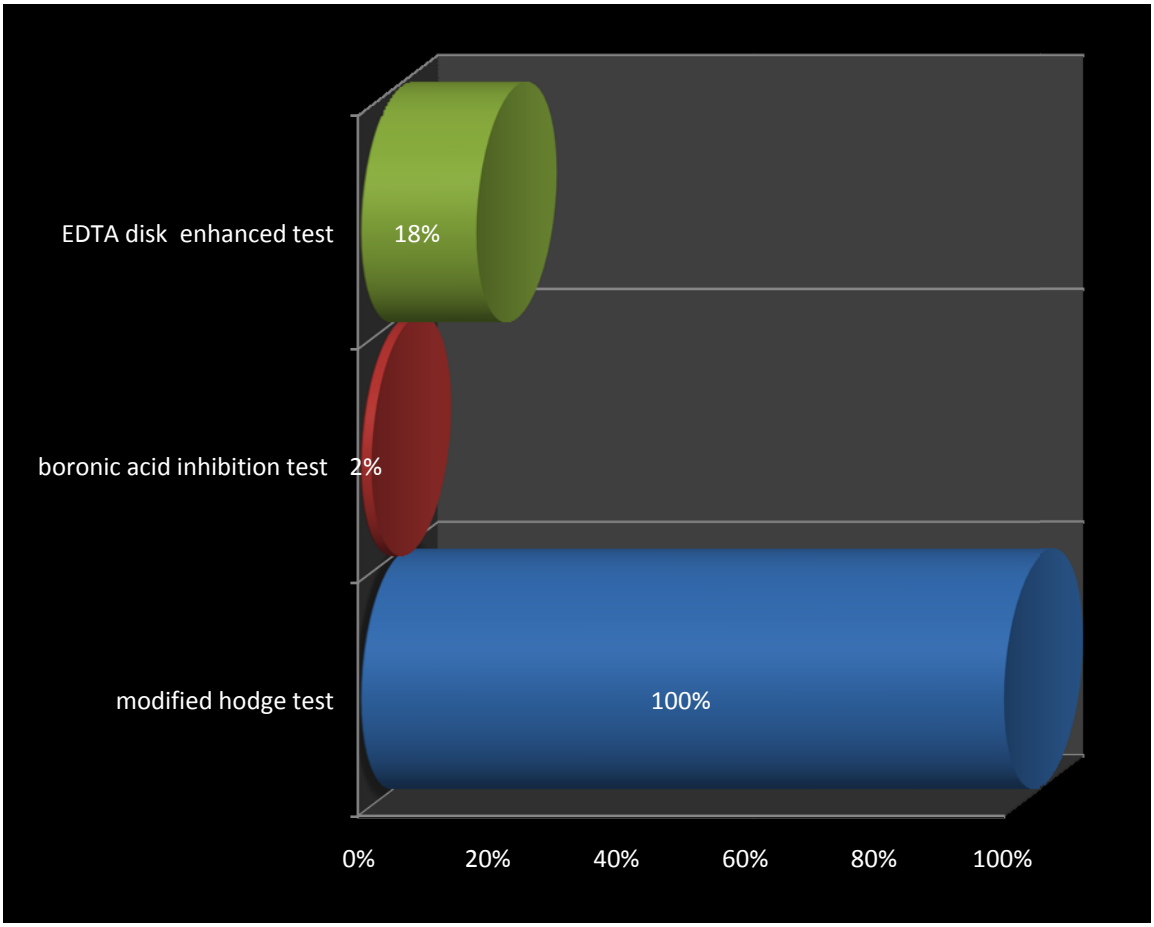


Figure 4. Modified hodge test.

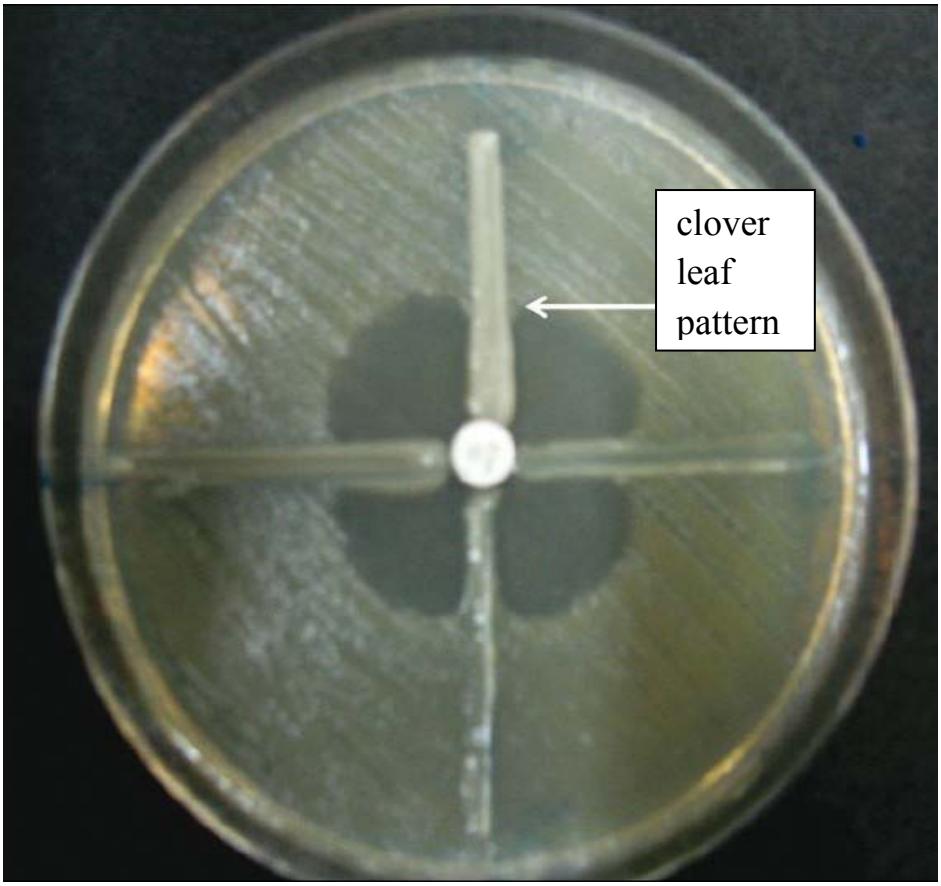
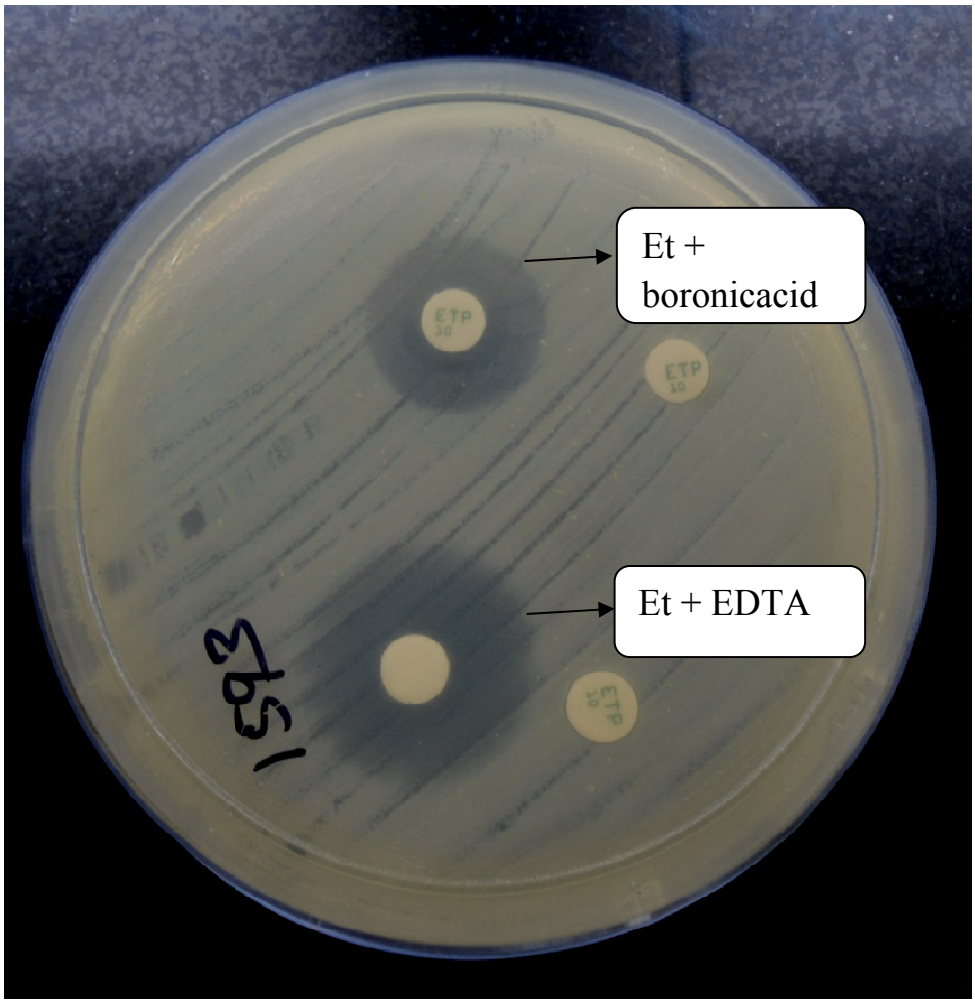


Figure 5 EDTA disk enhanced test and boronic acid inhibition test.



Distribution of OXA genes in the isolates:

Figure:6&7 OXA 51 and OXA 23 positive bands in gel electrophoresis.

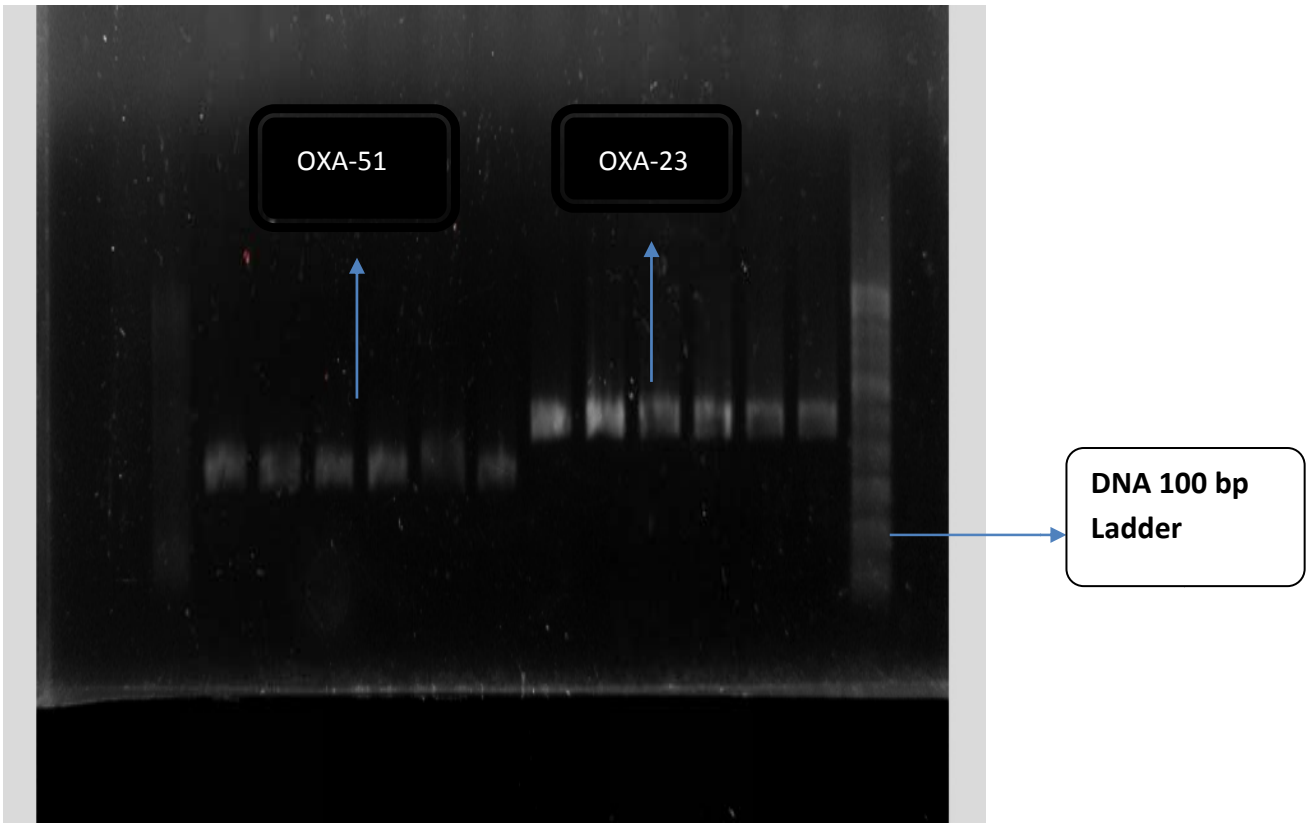
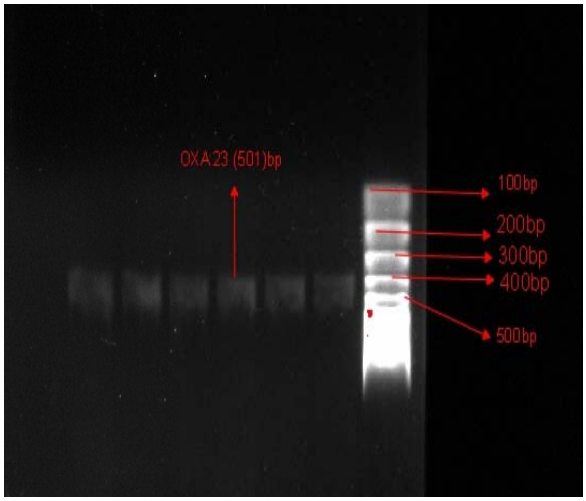


Figure :7



OXA-51 = 353bp.

OXA-23= 501bp.

Table12.DISTRIBUTION OF GENOTYPES AMONG THE ISOLATES

S.NO	OXA-TYPE	TOTAL no. of isolates tested	NO. OF POSITIVE ISOLATES
1	OXA-51	72	**72**
2	OXA-23	72	**67**
3	OXA-24	72	**00**
4	OXA-58	72	**00**

Of the total number isolates tested all were positive for the OXA 51 and OXA 23 was positive for 67 of the 72 isolates tested.OXA 51 was positive in all isolates conforming the identity.

The genes OXA 24 and OXA 58 was absent in all the strains tested.

Drug synergy test.

In random 10 isolates were take which were positive by modified Hodge Test and susceptible only to colistin was taken and underwent drug synergy test by checker board method with Rifampin amikacin sulbactam ciprofloxacin and Imipenem in combination with colistin.

The results were interpreted as synergy indifferent and antagonistic based on the∑ FIC values calculated

That is if the ΣFIC values

- ≤ 0.5 is interpreted as synergy
- 0.5 - 4 as indifference
- the values > 4 indicate antagonism.

Table 12 **Combination of colistin with sulbactam**

Isolate	∑Fic	Interpretation
Isolate 1	0.615	Indifferent
Isolate 2	0.725	Indifferent
Isolate 3	0.85	Indifferent
Isolate 4	0.091	Synergy
Isolate 5	0.122	Synergy
Isolate 6	0.185	Synergy
Isolate 7	0.122	Synergy
Isolate 8	0.56	Indifferent
Isolate 9	0.075	synergy
Isolate 10	Developed complete resistance	

That is if the ΣFIC values

- ≤ 0.5 is interpreted as synergy
- 0.5 - 4 as indifference
- the values > 4 indicate antagonism.

Table 13 **Combination of colistin with rifampin**

Isolate	∑Fic	interpretation
Isolate 1	0.455	synergy
Isolate 2	0.832	indifferent
Isolate 3	0.291	synergy
Isolate 4	0.787	indifferent
Isolate 5	0.437	synergy
Isolate 6	0.490	synergy
Isolate 7	0.483	synergy
Isolate 8	0.479	synergy
Isolate 9	0.093	synergy
Isolate 10	0.398	synergy

the ΣFIC values

- ≤ 0.5 is interpreted as synergy,
- 0.5 - 4 as indifference
- the values > 4 indicate antagonism.

Table 14 **Combination of colistin with ciprofloxacin**

Isolate	∑Fic	interpretation
Isolate 1	0.728	indifferent
Isolate 2	0566	indifferent
Isolate 3	0.66	indifferent
Isolate 4	0.66	indifferent
Isolate 5	0.66	indifferent
Isolate 6	0.83	indifferent
Isolate 7	0.389	synergy
Isolate 8	0842	indifferent
Isolate 9	0.615	indifferent
Isolate 10	1.00	indifferent

the ΣFIC values

- ≤ 0.5 is interpreted as synergy,
- 0.5 - 4 as indifference
- The values > 4 indicate antagonism.

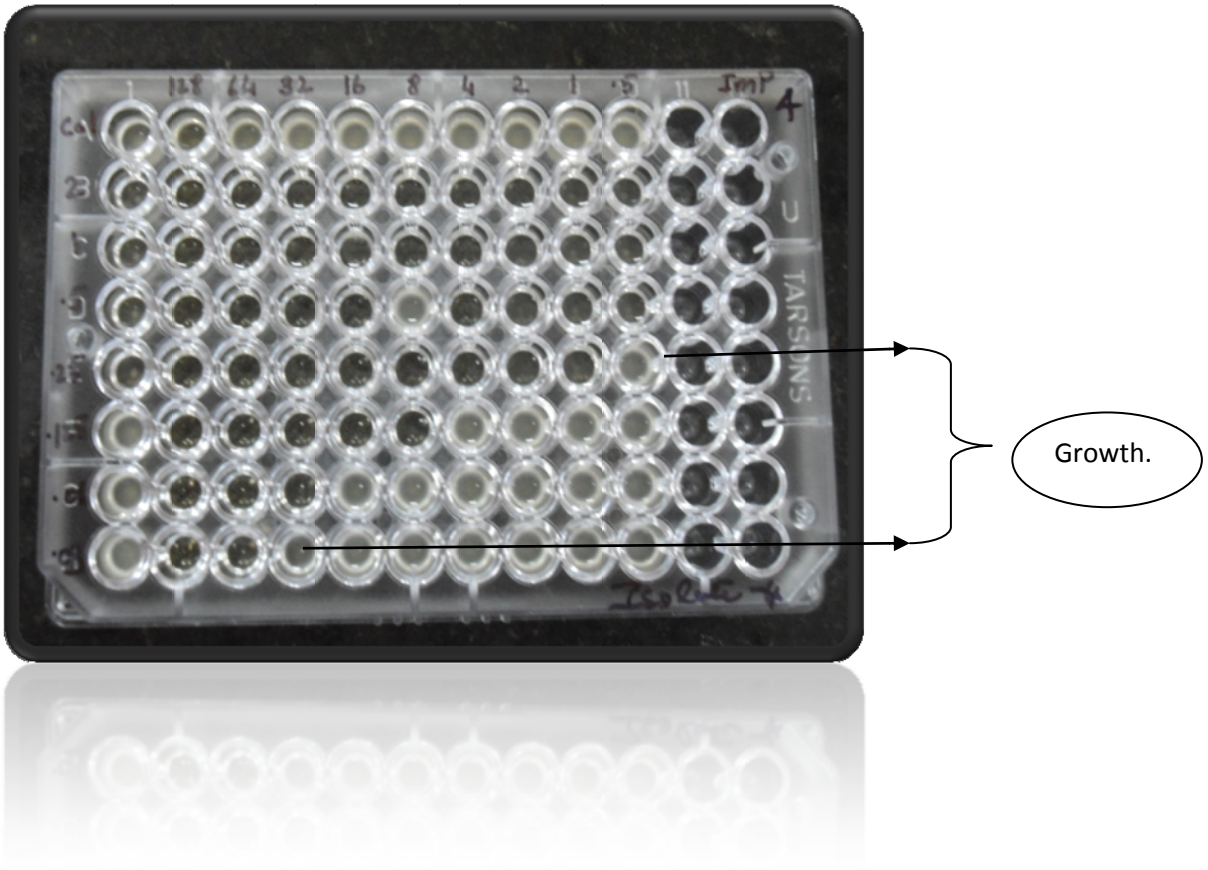
Table 15 **Combination of colistin with imipenem**

Isolate	∑Fic	interpretation
Isolate 1	0.122	synergy
Isolate 2	0.310	synergy
Isolate 3	0.56	indifferent
Isolate 4	0.185	synergy
Isolate 5	0.067	synergy
Isolate 6	0.56	indifferent
Isolate 7	0.122	synergy
Isolate 8	0.185	synergy
Isolate 9	0.125	synergy
Isolate 10	Developed complete resistance	

the ΣFIC values

- ≤ 0.5 is interpreted as synergy,
- 0.5 - 4 as indifference
- the values > 4 indicate antagonism.

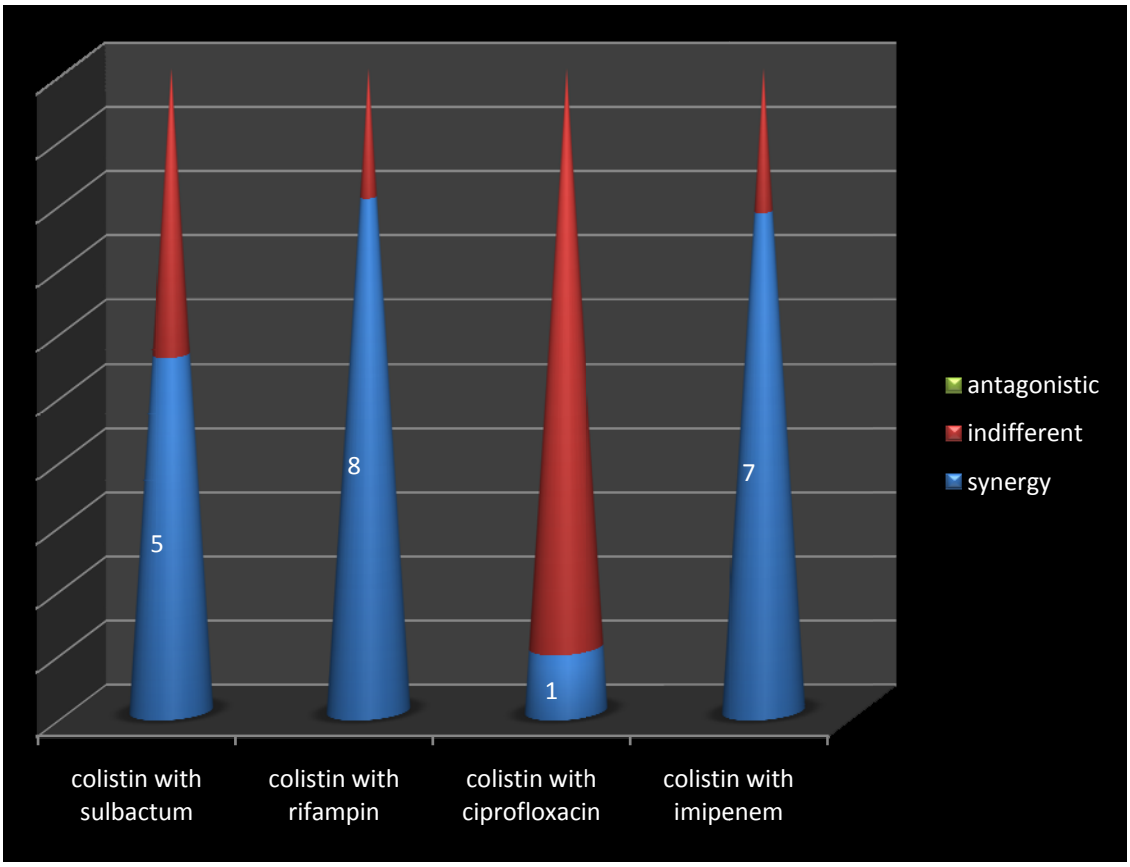
Figure 8 Microtiter plate used in Drug synergy tesing.



Combination of colistin with amikacin

The drug synergy between colistin and amikacin could not be determined as because the MIC of amikacin could not be determined (>1024mg/ml)

Figure 2



Based on the ∑FIC values the combination of colistin with rifampin and imipenem had maximum number synergy 8 and 7 isolates of the total ten were found to be synergy

In sulbactam combination 5 isolates showed synergy while four remained indifference. As the test with sulbactam and imipenem was done last isolate no.10 developed resistance to colistin so the results could not be determined.

The combination of ciprofloxacin and colistin showed the least effect as only one showed synergy and the rest of the isolates were indifferent.

6. DISCUSSION

DISCUSSION

The genus *Acinetobacter* which is a gram negative nonmotile nonfermenting coccobacilli that has developed into a significant pathogen in the healthcare institution. They mostly infect the critically ill patients in the intensive care units in the last 20 years it has emerged as deadly pathogen with their ability to acquire resistance, making it one of the most threatening bacteria in the antibiotic era.

In our study the prevalence of the pathogen was found to be 6.8%which was next only to other most common pathogen like *Escherichia coli (64.89%) Pseudomonas aeruginosa(9.8) candida (9.6) kiebsiella pneumoniae (8.6%) and Staphyloccus aureus(7.6)* in our lab. This was found to slightly lower when compared to study done in north India by Namita Jaggi et al[80] (7.9%) but few reports indicated less prevalence in India (3.6%) and abroad (<1%), very high prevelance was observed in USA[67] (15%) .

The most common site of isolation of the pathogen was found to be respiratory tract (66%) samples and was followed by wound swab (24%) and blood (8%) [80] in our study similar pattern was observed in other studies (56.9%). A study from Delhi[68] observed that most of the *Acinetobacter* were from wound however major source of this bacteria was respiratory tract in the western countries[82].

Acinetobacter baumannii has emerged as one of the important pathogen due to its ability to resist beta-lactams, including carbapenems by various antimicrobial resistance mechanisms. In this study about 58.02% of the strains were carbapenems resistant. Other studies from North and South India (90%) reported higher incidence of carbapenem resistance, however very low incidence was reported from AIIMS in 2010.

Higher incidence of carbapenem resistancewere reported from countries like Greece and Italy (>90%)[81] USA (44.8%) and Middle East (25.3%)[67].

All isolates of *Acinetobacter baumannii* in our study were sensitive to colistin and polymixin B majority of the isolates (76%) were sensitive to Tigecycline. Susceptibility to Amikacin chloramphenicol ofloxacin co-trimoxazole was all less than 20%.

Multidrug resistant organism is defined as an isolate that is not susceptible to one or more agents in at least three antimicrobial categories. Multidrug resistance of 73% was observed in our study which was higher than reports from Delhi (41.5%)[68] multidrug resistance incidence in *Acinetobacter baumannii* isolates from developed countries were less example 21% in usa and <5% in Norway[80].

Pandrug resistance is defined as non-susceptibility to all agents in all antimicrobial categories for the isolate. Pandrug resistance was found to be <1% of our isolates which was less than other studies from India (3.5%) and West (<4%)[80].

Out of 58.02% of carbapenem isolates of *Acinetobacter baumannii* in our study Modifed hodge test was positive in all isolates similarly finding to modified hodge test positive was observed by Aparna Shivaprasad[82] et al. Mahajan G et al found only 46.7%. This result indicates

the probability of non existence of Nonenzymatic causes of carbapenem resistant example efflux pump in our study.

EDTA disk enhanced test is used to identify metalobetalactamases production in carbapenem resistant bacteria among the 72 isolates carbapenems resistant isolates in our study were positive by 18.08% EDTA disk enhancement test indicating the production of beta lactamase higher MBL production rate 67.5 % was observed in other studies[83].

Boronic acid inhibition test is used for the identification of AmpC production among the carbapenem resistant isolates 2.12% of the isolates were positive which was lower in other studies which observed at 16%.

MOLECULAR EPIDEMIOLGY OF CARBAPENEMASES

The beta-lactamases are classified into 4 different molecular groups A, B, C and D based on the amino acid sequence. The class D beta-lactamases referred to as oxacillinases as the can hydrolysis oxacillin the

first reported class D beta-lactamases was seen in *Acinetobacter baumannii* called as OXA 23 also know as ARI-1 *A. baumannii* and *A. radioresistens* both have been known to possess class D betalactamase naturally and *Acinetobacter* species may be a reservoir for class D genes.

From various geographic areas OXA 51 was isolated from *Acinetobacter baumannii* and were chromosomally located and found to be intrinsic to this species.

In our study the gene OXA 51 was found in 100% of the isolates. The OXA 51 which chromosomally located in *Acinetobacter baumannii* is naturally occurring in this organism. This has been reported in various article and studies all over the word including India. OXA 51 present inherently in all *Acinetobacter baumannii* was identified in all our isolates conforming the identity of the *Acinetobacter baumannii* isolates.

The gene OXA 23 was found in 63 isolates (93.05%) and also found to be co-existing with OXA 51 In a similar study by Amudhan SM et al in 2011 found OXA 23 in 89.09% of their isolates, presents of OXA 23 and it co-existed with OXA 51 in 83% of their isolates[84]. In some the carbapenem resistant isolates (20.89%) of *Acinetobacter baumannii* both

OXA 23 and MBL were seen indicating more than one mechanism of resistance.

The gene OXA 24 and 58 were not found in any of our isolates where as Amudhan et al observed in only of their isolates carrying OXA 24 and OXA 58. In a similar study from North India by Niranjan D K[85] et al in the year 2013 OXA 58 and OXA 24 were mot present in their isolates. In all the above studies showed that OXA 51 and OXA 23 are the most prevalent OXA types in *Acinetobacter baumannii.*

Drug synergy testing:

Earlier *Acinetobacterbaumannii* infections have been treated with mostly third generation cephalosporins and extended spectrum penicillins have been used for the treatment. In the case of severe infection penicillin with beta-lactam inhibitors and cabapenem with combination with aminoglycosides have been used. At the end of the last century *Acinetobacter* and other bacteria became associated with carbapenemase production and were resistant to two or three group of drugs including carbapenems. polymixins are only group of drugs that are used for its treatment of such isolates.

In last few years antibiotic combination has been proposed for the treatment and management of these infection caused by such organisms,In combination therapy the right combination of the drugs is very important and also determining the dose of both the main drug and the adjuvant drug to achieve the PK/PD so as to achieve the maximum efficiency and decrease the chance of resistance and to decrease the toxicity.

Combination of colistin with sulbactam showed 55.55% (5/9) synergy in our study which was similar to those observed by Karaoglan 53.3% synergy. A clinical study in Brazil also showed better clinical outcome in 73.8% of patients with this combination therapy with colistin and sulbactam[86].

Colistin with Rifampin combination showed 80% (8/10) synergy in our study, where as song et al observed 100% synergy with this combination they also found positive results in animal mouse model which showed 6 day survival rate with this combination[87].

Combination of colistin with ciprofloxacin

Cagla Bozkurt[88] et al observed 45% (9/20isolates) synergy with this combination of colistin with ciprofloxacin where as 10% synergy was only seen in our study.

Combination of colistin with imipenem

We observed 77.77% synergy with imipenem when combined with Colistin Yoon et al[89] found much higher (100%) synergy with this combination whereas no synergy in this combination by others likes Wareham et al.

The drug synergy between colistin and amikacin could not be determined as because the MIC of amikacin could not be determined (>1024mg/ml) very high resistance.

One strain developed resistance to colistin after preservation and revival indicating the existence of hetro-resistance which has to be proved similar observation was made by Li J, Rayner et al[90].

Difference in synergistic effects of particular combination of drugs in different studies where compared to each other may be due to clonal difference in *Acinetobacter baumannii*.

7. SUMMARY

Summary.

- A total of 163 isolates of *Acinetobacter* species were characterized by both manual phenotypic method and vitek automated identification method.

- *Acinetobacter baumannii* was the most common species(45) followed by *Acinetobacter* species (13), *Acinetobacter loffii(3)* and *Acinetobacter haemolyticus(2).*

- Antibiotic susceptibility testing was done by kirby bauer disk diffusion method and vitek automated method.

- Pandrug resistance was seen in <1% of the isolates

- Multidrug resistant and only 73% isolates

- Carbapanem resistance was seen in 58.02%of the isolates.

- Modified Hodge Test was positive in 100% of the isolates

- EDTA disk enhanced test was positive in 17% of the isolates indicating the metalobetalactamase production.

- Boronic acid test was positive in 2% of the isolates indicating AmpC production.

- Molecular characterization of OXA genes were performed in all isolate which were positive by MHT

  OXA 51 was seen in 100% of the isolates, conforming the identity of the isolates.

  OXA 23 was seen in 93.05% which was also seen in (20.89%) isolates that were MBL positive

  OXA 24 and OXA 58 were not seen in any isolate.

- Ten randomly picked isolates of colistin sensitive *Acinetobacter baumannii* were subjected to Drug synergy test by checker board method. We observed maximum synergy in colistin with rifampin combination(80% and imipenem (77.77%), only 55.55% synergy was observed with sulbactam and very poor synergy was observed with ciprofloxacin(10%).

8. CONCLUSION

CONCLUSION

Acinetobacter baumannii is one of the important nococomial pathogen which showed high incidence of carbapenem resistance (58.03%). Most common OXA genes found in carbapenem resistant *Acinetobacter baumannii* were OXA 51 and OXA 23. Metalo-betalactamese was seen in 18.08% of isolates Best synergy was observed with colistin and rifampin (80%) followed by imipenem (77.77%) .

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